Cell death in platelets and megakaryocytes: implications for the maintenance of thrombostasis

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Abstract

Apoptosis is a complex and ubiquitous physiological program for the deletion of cells in which caspases typically direct morphological and biochemical changes leading to safe clearance by phagocytes. However, a growing body of evidence now suggests that not all forms of cell death require caspases, and that caspases themselves can participate in cellular events that do not ultimately lead to the demise of the cell.

Maintenance of platelet number, thrombostasis, is an essential element for the preservation of haemostasis, and thus the existence of a complex metazoan. The platelet is a small anucleate cell found only in the circulation and is primarily responsible for maintaining the integrity of the vascular system. This is achieved by aggregating to one another and to exposed ECM to form part of the physical barrier to prevent blood loss, and by directing repair to the damaged endothelium. Platelets are formed in the bone marrow by a poorly understood cellular fragmentation of their progenitor, the megakaryocytes, and thus megakaryocyte proliferation has been the focus of study in understanding the control of platelet numbers.

This thesis reports that thrombostasis is potentially maintained by the relative level of two opposing and different cell death programs within megakaryocytes and platelets. Firstly, platelet formation from megakaryocytes occurs by a unique, compartmentalised form of caspase-dependent apoptosis that results in the formation of multiple functional anucleate progeny. However, whilst the megakaryocyte cell body contains active caspases and displays nuclear condensation and fragmentation typical of apoptosis, the forming platelets retain functional mitochondria, do not contain active caspases, and are not phagocytosed, emphasising an atypical compartmentalised mitochondrion-independent apoptotic program. Platelet formation from megakaryocytes could be significantly augmented by ligation of the Fas death receptor, and reduced by treatment with caspase inhibitors.

Secondly, this thesis reports a constitutive but caspase-independent program for the specific phagocytic clearance of intact effete platelets. Platelets aged *in vitro* exhibited increased expression of proapoptotic Bak and Bax, underwent diminution of function, and displayed cytoplasmic condensation and plasma membrane changes that lead to recognition by phagocyte scavenger receptors. However, although platelets contained the effector caspase-3 they lacked caspase-9, a key component of the apoptosis initiator complex the apoptosome. Intriguingly, megakaryocytes contained both caspase-3 and -9, suggesting sequestration of the enzyme by the progenitors during platelet formation, again underscoring the compartmentalised death of megakaryocytes and thus explaining the inability of platelets to activate caspases during constitutive death, and hence their commitment to undergo a caspase-independent death.

In summary, this study demonstrates that platelet numbers may in part be controlled by the level of megakaryocyte apoptosis within the bone marrow, and hence number of platelets released into circulation, and inversely by a constitutive death program within the platelet leading to biochemical changes resulting in their clearance and hence removal from the circulation. Although limited in its *in vivo* relevance, the study also emphasises that diseases of thrombostasis may reflect abnormalities in the control of cell death, and hence presents the potential for novel strategies of therapeutic intervention.

Declaration

I hereby declare that this thesis has been composed solely by myself and has not been accepted in any previous application for candidature for a higher degree. All work presented in this thesis was, unless acknowledged, initiated and executed by myself. All sources of information in the text have been acknowledged by reference.

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Dedication

This thesis is dedicated to my parents, Mary and David, and my grandmother, the late Gertrude Chapman

Abbreviations

2D-IB	Two dimensional electrophoresis immuno blotting
Ab	Antibody
ADP	Adenosine diphosphate
ALLN	acetyl-leucyl-leucylnorleucinal
APAF-1	Apoptotic protease activating factor-1
APS	Ammonium persulphate
ATP	Adenosine triphosphate
Bowes	Bowes melanoma cell
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
CARD	Caspase recruitment domain
CD	Cluster of differentiation
dATP	Deoxy-adenosine triphosphate
DD	Death domain
ddH_2O	Distilled deionised water
DED	Death effector domain
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulphoxide
ECM	Extra cellular matrix
EDTA	ethylenediaminetetraacetic acid
FACS	Fluorescence activated cell sorter
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
Gp	Glycoprotein
HBSS	Hank's balanced salt solution
HRP	Horse radish peroxidase
IFN	Interferon
IGF-1	Insulin-like growth factor one
IL-	Interleukin
Jurkats	Jurkat T cells
K/O	Knock out (ablated gene)
kD	Kilodalton
LDH	Lactate dehydrogenase
mAb	Monoclonal antibody
mCCCP	Carbonyl cyanide m-chlorophenyl-hydrazone
MDMs	Monocyte-derived macrophages

MK(s)	Megakaryocyte(s)
MΦ	Macrophage
OCS	Open canalicular system
pAb	Polyclonal antibody
PARP	Poly (ADP ribose) polymerase
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PE	Phycoerythrin
pI	Isoelectric point
PI	Propidium iodide
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethylsulfonyl fluoride
PPP	Platelet poor plasma
PPPDS	Platelet poor plasma derived serum
PRP	Platelet rich plasma
PRPDS	Platelet rich plasma derived serum
PS	Phosphatidylserine
PTP	Permeability transition pore
PVDF	Polyvinylidene difluoride
Rf	Relative distance of migration
S.D.	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SSC	Side scatter
TBS	Tris-buffered saline
TEM	Transmission electron microscope
TEMED	N,N,M'N'-tetramethylethylenediamine
TGF-β	Transforming growth factor-beta
TNF	Tumour necrosis factor
TPO	Thrombopoietin
Tris	Tris(hydroxymethyl)aminomethane
vWF	von Willebrand Factor
w/	With
w/o	Without
zDEVD-fmk	Carbobenzoxy-Asp-Glu-Val-Asp-fluoromethylketone
zLEHD-fmk	Carbobenzoxy-Leu-Glu-His-Asp-fluoromethylketone
zVAD-fmk	Carbobenzoxy-Val-Ala-Asp-fluoromethylketone
$\Delta\psi M$	Inner mitochondrial membrane potential

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Chapter 1 - Introduction

Apoptosis and cell death

1.1 Historical Background

There is an ancient samurai saying from the Hagakure that says, "once we are in the land of the living, we will eventually die" (Tsunetomo 1716). This is of course true not only for humans but also for the cells from which we are made. We are all derived from one single egg, which after repeated cell division becomes the billions of cells that constitute our bodies. During this process many damaged, unwanted, or harmful cells must be removed in a safe and efficient way. The study of cell death - including apoptosis - now holds near to 100,000 publications, but although biologists have assumed the field to have first appeared in the 1970s, as with most science we have spent much of our time reinventing the wheel, the true "birth" of cell death being in the late nineteenth century. One of the earliest recorded investigations comes from the German development biologist Vogt who characterised the natural death of notochordal and cartilaginous cells during development (1842). However not until forty years later was unmistakable evidence of what we now refer to as apoptosis seen, with Flemming's study of regressing ovarian follicles containing hand drawn microscope transcriptions of cells showing clear margination of chromatin (1885). After a promising start the field changed direction to study the removal of cell corpses, with the Russian émigré Ilya Mechnikov winning a Nobel Prize for his discovery of phagocytosis (1908). Perhaps in some ways this reflects the paradigm shift seen at present with the final stage, and as many would argue the most important step of apoptosis, the phagocytic clearance, emerging to take centre stage once again.

However, it was not until the early1970s when the true extent of the implications of apoptosis, and at that time coined "shrinkage necrosis", was appreciated. Following the union of "the grandfathers of apoptosis", John Kerr, Andrew Wyllie and A.R. Currie in Aberdeen, the three published their seminal paper (1972). This described the key observations that dying cells were in fact following a specific and conserved pathway, and most importantly that the program was a biological phenomena "as natural as mitosis, secretion, or glycolysis". In addition, they extended

their findings to the wide-ranging implications that the regulated kinetics of such a program would have on the control of an animal's total cell population - tissue homeostasis. Following consultation with James Cormack, a professor of Ancient Greek, the term "apoptosis" was coined, deriving from a Greek word describing the "falling off" of petals from a flower, or leaves from a tree. Given a publication on the role of *defender against apoptotic death* protein (DAD1), expressed in mammalian and plant cells, which was shown to be down-regulated during senescence of flower petals (Orzaez and Granell 1997), the term apoptosis represents either extreme serendipity, or immense foresight by the authors in the extent to which apoptosis plays a role.

1.2 Basics of Cell Death

1.2.1 Apoptosis vs. Necrosis

Until the realisation that cells could engage a natural cell death program, death was thought to occur as a result of a pathological condition. This form of "misguided" cell death, termed necrosis, tends to affect "fields" of cells and occurs in response to a number of noxious stimuli such as ischemia, hypothermia, hypoxia, complement attack, metabolic poisons, and direct mechanical cell trauma (Wyllie 1980). An understanding of the mechanisms and processes of necrosis is undoubtedly required to appreciate the need for such a distinct and defined program as apoptosis. Early necrosis proceeds through swelling of the cytoplasm and organelles, especially the mitochondria. When hydrostatic pressure reaches a critical point, structural integrity cannot be maintained and the cell lyses releasing its contents. Due to this release inflammation develops in surrounding tissue in response to the released contents (Wyllie *et al* 1980). Apoptosis on the other hand tends to affect single scattered cells in response to many environmental and developmental prompts such as genotoxic insult, factor deprivation, virus infection, glucocorticoid treatment, and heat shock (Wyllie 1980). Regardless of the wide-ranging set of insults, apoptosis proceeds in a predictable fashion with a conserved chain of observable morphological changes. The cell's cytoplasm begins to condense along with margination of the chromatin, with many cell types undergoing blebbing of the plasma membrane. Eventually, *in vitro,* the cell fragments into membrane bound "apoptotic bodies"

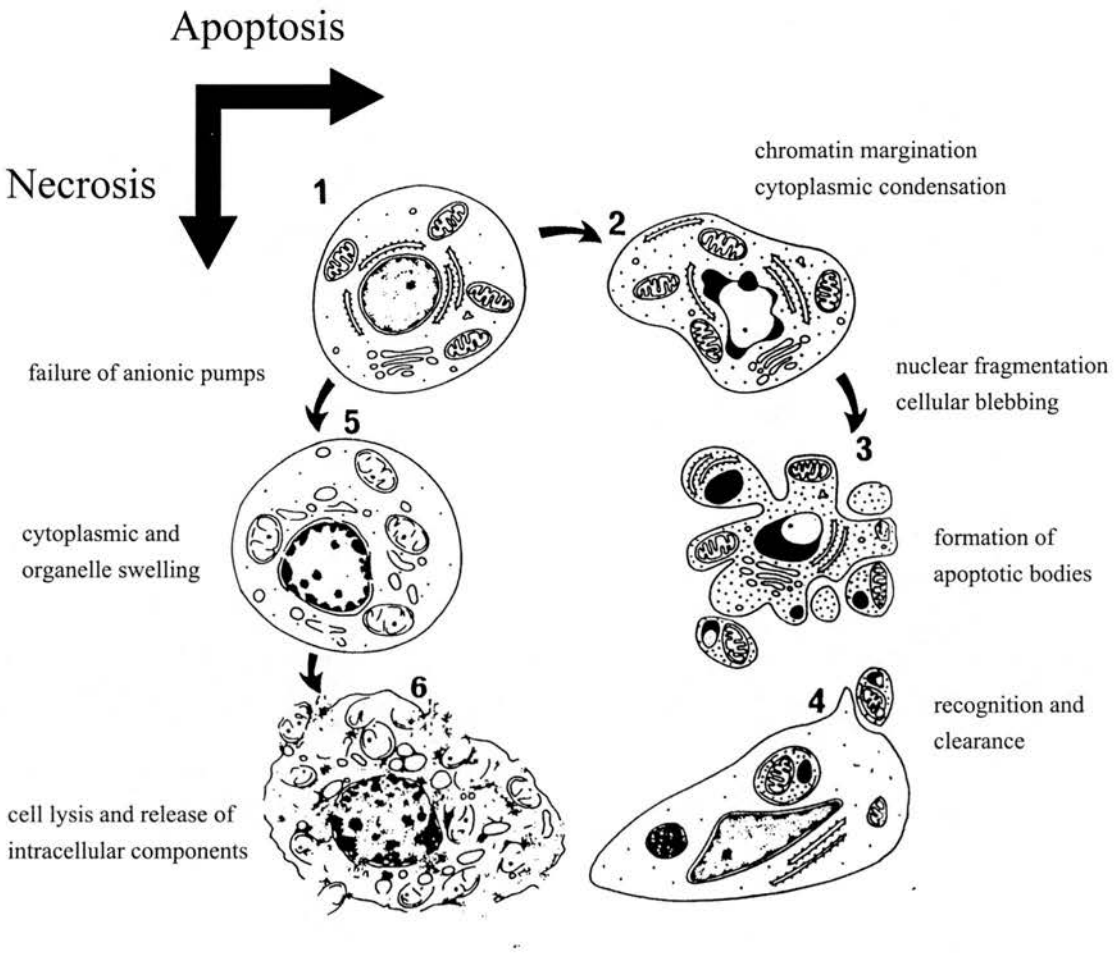

Figure 1.1: The morphological features of apoptosis and necrosis. Adapted from Kerr *et al* (1972)

containing intact organelles and nuclear material. These are recognised, ingested, and degraded by neighbouring cells - a process named phagocytosis. A basic schematic of these two processes are outlined in Figure 1.1. Since apoptosis remains a relatively contained process with no leakage of cytoplasmic components, and hence little bystander cell death and inflammation, the process remains fairly inconspicuous within tissue histology, which is thought to reflect the true efficacy with which phagocytes remove apoptotic cells *in vivo*.

1.2.2 Better to be dead than wrong

Given the advantages of a refined form of cell death such as apoptosis compared to necrosis, one might ask what is the point of naturally occurring cell death at all. Cells that die during normal development and tissue homeostasis have been shown to fit into five broad categories, according to the extensive work by Ellis *et al* (1991): (1) cells that have no function and thus can be eliminated, (2) cells that are generated in excess, some of which may be eliminated, (3) cells that develop incorrectly, (4) cells that have fulfilled their function, and (5) cells that are harmful and must be eliminated to protect the organism. From this we can see that apoptosis plays important roles ranging from embryogenesis (i.e. sculpting of organs and tissues: "cell division forms the clay, whilst cell death sculpts the clay into the desired form", in the words of Michael Hengartner, personal communication), to tumour regression by acting as "anti-cancer" machinery (Neubauer *et al* 1996), and particularly striking as the mechanism for clonal deletion of undesirable T-cells activated to self-antigens (Wu *et al* 1993). Therefore it could be said that apoptosis is the "workhorse" to attain and maintain a normal organism in homeostasis.

1.2.3 Away from the nucleus

As apoptosis is an active conserved chain of events it was believed that RNA and protein synthesis would be required, in contrast to the passive "accidental" death by necrosis (Schwartzman and Cidlowski 1993). In fact, initiation of apoptosis by many stimuli occurs in the presence of protein or RNA synthesis inhibitors, Fas-mediated being the archetypal case (Yonehara *et al* 1989; Itoh *et al*

1991). In addition, it was shown many biochemical and morphological changes could take place in enucleated cells (Jacobson *et al* 1994; Schulze-Osthoff *et al* 1994), leading to the key conclusion that all of the cell death machinery required is ready within the cytoplasm of most cells, and could therefore proceed without any need for nuclear factors (Weil *et al* 1996). It could be considered that these key findings helped "redirect" the field away from the nucleus, with its easily visualised morphological changes, to the cytoplasm and other organelles to search for more distinct biochemical changes. Much of the field of cell death has concentrated on the mechanisms of caspases and their activation by the mitochondrial pathway, however many other organelles and cellular components have been implicated to undertake important roles in cell death. Specifically, the Golgi apparatus (Bennett *et al* 1998), lysosomes (Ishisaka *et al* 1998), the cytoskeleton (Brancolini *et al* 1997), the endoplasmic reticulum (Ng *et al* 1997), the nucleolus (Stegh *et al* 1998), the plasma membrane (Fadok *et al* 1992a), etc, etc. Looking at a list like this can only bring one conclusion to mind. Organelles should not be singled out for their relative importance in cell death, since overall the entire cell is subjected to and must participate in the changes ultimately leading to its safe self-degradation and removal.

1.3 Mechanisms of Cell Death

The rapid advances in biochemical techniques and support technology over the last decade have allowed a rapid advancement in the elucidation of the molecular machinery responsible for implementing biochemical changes during cell death. A myriad of publications have documented the importance of a number of key molecular players and proposed hypothetical models incorporating them, including the Bcl-2 family members and their interaction with the mitochondria, caspases, endonucleases, kinases, death receptors, oncogenes, and tumour suppressors. Many have divided the death pathway into hypothetical stages. For example Kroemer has suggested three stages comprising pre-mitochondrial, mitochondrial, and post-mitochondrial (1997a), to clearly over-emphasise the central role of this organelle, but given the number of possible alternative pathways this represents somewhat of a bias. Given the somewhat unusual scope of the current thesis a more holistic and

liberal division is proposed, also encompassing the consequences of death. Therefore death is divided into three stages: initiation, execution and resolution.

Initiation represents the triggering of death by a wide range of stimuli followed by the "decision" to die and commitment to death. What ultimately represents the final On/Off switch, if such a point exists, is still not known, although many changes can be considered biochemically irreversible such as release of cytochrome-C from the mitochondria (Liu *et al* 1996), or the clearly irreversible proteolytic cleavage of the caspases (Thornberry *et al* 1992). The execution phase corresponds to the degradation of a discrete subset of signalling, transcriptional, and structural proteins, as well as the genetic material itself, by a cascade of proteases and eventual endonuclease activation. Finally, during the resolution stage cells expose or alter cell surface markers to states or conformations that ultimately result in recognition, uptake, and degradation by neighbouring cells, a process termed phagocytosis. The entire chain of events occurs without the release of intracellular components from the dying cell, and hence prevents a deleterious inflammatory response. In the following sections a more in depth focus on these processes will be presented.

1.4 Caspases – Homicidal Proteases

1.4.1 A family of cysteine proteases

Caspases are cysteinyl-proteases with a specificity to cleave proteins to the carboxy-side of aspartic acid residues (Thornberry *et al* 1992; Nicholson *et al* 1995), and are recognized as the major executioners of cell death responsible for driving the apoptotic cell death phenotype. The name caspase derives from the cysteine residue within the catalytic diad active site and substrate specificity, hence *C*ysteine *aspases*. This ability to cleave to the carboxyl-side of aspartic acid is extremely unusual for proteases allowing caspases to function with high specificity during cell death*, and hence helps protect against non-specific protease cleavage initiating cell death (Kidd *et al* 1998; Thornberry

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* The only other eukaryotic protease known to have a similar specificity is the serine protease granzyme B, a mediator of granule-dependent cytotoxic T lymphocyte mediated apoptosis.

and Lazebnik 1998). This specificity is also reflected in the observation that apoptosis is not accompanied by indiscriminate protein degradation, but rather the select cleavage of a subset of proteins in a coordinated manner. There are currently fourteen mammalian caspases, including two murine family members, caspase-11 and -12, for which human homologues have yet to be found (Thornberry and Lazebnik 1998). In addition to the primary specificity is a requirement for at least three amino acids to the amino side of the cleavage site, known as the P_2-P_4 positions. In contrast to the stringent P_1 requirement, the P_4 position was identified as the next most important determinant in defining the distinct substrate preferences of the various caspase sub-species (Thornberry *et al* 1997). From this caspases can be divided into three major specificity groups. Group I enzymes (caspase-1, -4, and -5) prefer the sequence WEHD but may be considered "promiscuous" compared to others. Group II enzymes (caspase-2, -3, and -7) favour DEXD, and display high selectivity. Group III enzymes (caspase-6, -8, -9, and -10) utilise the consensus sequence (L/V)EXD (Table 1.1). This broadly categorises them according to whether they function as initiators of a caspase cascade, group III, as effectors, group II, or are involved in inflammatory processes, group I.

Specificity group	P_4-P_1 specificity	Consensus	Proposed function
Group I			
Caspase-1	WEHD		maturation of
Caspase-4	(W/L)EHD	WEHD	pro-inflammatory
Caspase-5	(W/L)EHD		Cytokines
Group II			
Caspase-2	DEVD		effectors, i.e. cleavage
Caspase-3	DEVD	DEXD	of DXXD-containing
Caspase-7	DEHD		"death" substrates
Group III			
Caspase-6	VEHD		initiators, i.e. cleavage
Caspase-8	LETD	(I/V/L)EXD	of group II caspases
Caspase-9	LEHD		and non-DXXD substrates

Table 1.1: Specificities and proposed biological functions of caspases. X represents any amino acid. Adapted from Thornberry *et al* (1997).

Interestingly, all amino acids can be divided into aromatic, aliphatic, acidic and basic. Taking this, the amino acid in the P_4 position of all group I caspases is an aromatic, all group II have acidic at P_4, whilst all group III caspases have aliphatic amino acids at P_4. Assuming this is not an

incredible coincidence, could we be missing an entire family of caspases with basic residues at the P_4 position? Analysis of the SwissProt database for the sequence (H/K/R)EXD reveals many potentially interesting substrates for such putative caspases, including many membrane specific proteins, including most members of the ABC transporter family.

1.4.2 Activation by proteolysis

The majority of caspases are found as latent proforms within the cytoplasm, and are usually converted to an active enzyme by proteolytic processing. This can occur by either another protease or by autocatalysis, triggered by binding of cofactors or removal of inhibitors (Thornberry and Lazebnik 1998), and hence allows the accumulation of large amounts of precursor in advance that can be activated on demand. All pro-caspases contain three domains: an NH_2 terminal domain, a large subunit of ~20kDa, and a small subunit of ~10kDa. Activation occurs by sequential processing between the subunits (Thornberry and Lazebnik 1998). Initially the enzyme is cleaved into large and small subunits, followed by removal of the NH_2 domain from the large domain. The large and small domains form a heterodimer that, as evidenced by the crystal structures for active caspase-1 and -3, associates two heterodimers to form a tetramer containing two independently functional active sites (Rotonda *et al* 1996). The large and small subunits are intimately associated and both contribute residues to form the active site. This specific arrangement of all procaspases conveys two important mechanistic implications. Firstly, the NH_2 domain displays marked variation in sequence and length between caspases and is involved in the regulation of activation. Secondly, all cleavage sites within the pro-enzymes represent caspase consensus sequences, explaining the premise that these enzymes are activated either autocatalytically, or in a cascade by enzymes with similar specificities. This led to postulation, and later proof, that a hierarchical relationship must exist between "initiator" caspases, containing much larger NH_2 domains, and "effector" caspases that result in the cell disassembly.

1.4.3 The role of caspases in cell death

The notion of a proteolytic cascade is central to the activation of caspases and hence control of cell death, providing multiple points of regulation to prevent unwanted activation of effector caspases. In fact, given that caspases mediate such a drastic process as cell death the complexity of their regulation rivals that of the complement or coagulation cascades. Multiple apoptotic stimuli result in the activation of initiator caspases, which in turn activates effector caspases. Different initiator caspases mediate distinct signals from apoptotic stimuli. For example, caspase-8 mediates activation of effector caspases in response to Fas ligation (Ashkenazi and Dixit 1998), whilst caspase-9 is involved in death induced by cytotoxic agents (Hakem *et al* 1998) explaining how a range of stimuli can induce the same biochemical and morphological changes. The ultimate objective of effector caspase-mediated proteolysis is the disassembly of the cell, and to mediate changes resulting in the eventual safe clearance by phagocytes. Although over one hundred different caspase substrates have been identified (Stroh and Schulze-Osthoff 1998), mainly through protein database analysis of caspases consensus recognition residues, only a small proportion of these are understood in their relationship to cell death at any level. Caspase-mediated cleavage of substrates can be considered to result in either a gain of function, such as the loss of a regulatory domain from a catalytic subunit, or a loss of function, such as cleavage to inactivate an enzyme or the disassembly of structural proteins.

One role of caspases is to inactivate proteins that serve to protect living healthy cells from effecting apoptosis. The archetypal example of this is cleavage of ICAD, an inhibitor of the caspase-activated deoxyribonuclease (CAD) responsible for oligonucleosomal DNA fragmentation. In nonapoptotic cells CAD is present as an inactive complex with ICAD. During apoptosis the caspase cascade activates caspase-3 resulting in cleavage and inactivation of ICAD, allowing CAD to function as a DNAse (Liu *et al* 1997; Sakahira *et al* 1998). Several members of the Bcl-2 family of anti-apoptotic regulators are also cleaved by caspases (Xue and Horvitz 1997; Cheng *et al* 1997). Caspase cleavage not only prevents their anti-apoptotic capability, but also produces fragments that actually promote apoptosis, effectively giving positive feedback to ensure the inevitability of death (Cheng *et al* 1997).

The major morphological changes witnessed during apoptosis are also caspase mediated. Destruction of the nuclear lamina, a rigid structure underlying the nuclear membrane and involved in chromosomal organisation, occurs through cleavage of lamins at a single site. Lamins represent intermediate filaments that polymerise to form nuclear lamina, which on cleavage cause the structure to collapse and contribute to chromatin condensation (Lazebnik *et al* 1995; Rao *et al* 1996). Indirect reorganisation of cell architecture occurs through caspase cleavage of many proteins directly responsible for cytoskeletal regulation, including gelsolin (Kothakota *et al* 1997), focal adhesion kinase (FAK) (Wen *et al* 1997), p21-activated protein kinase (PAK2) (Rudel and Bokoch 1997), and fodrin/spectrin (Wang *et al* 1998). In general, cleavage of these proteins results in deregulation. However, in the case of gelsolin, a protein that severs actin in a Ca^{2+} controlled manner, cleavage produces a constitutively active fragment (Kothakota *et al* 1997).

The third major targets of the caspases are proteins involved in repair and homeostatic functions such as DNA-PK_{cs} (Casciola-Rosen *et al* 1995), U1-70K (Casciola-Rosen *et al* 1994), PKCδ (Emoto *et al* 1995; Song *et al* 1996). Taken together it can be seen that caspases act as the major "workhorses" of apoptosis, acting with swift military precision. They cut off contacts with surrounding cells, shut down DNA replication and repair, reorganise the cytoskeleton, interrupt splicing, disrupt nuclear structure, destroy DNA, fragment the cell into apoptotic bodies (Coleman *et al* 2001; Sebbagh *et al* 2001) and in many cases mediate surface changes, such as exposure of phosphatidylserine (PS), to allow phagocytic clearance (Fadok *et al* 1992a).

1.4.4 APAFs and the apoptosome – the intrinsic pathway

The intrinsic pathway is a term reflecting a cell death decision triggered internally in response to a number of cellular insults and stresses including growth factor withdrawal, chemical treatment (e.g. staurosporine), or DNA damage to the cell. Activation of this death pathway is thought to converge on the mitochondria, which has been demonstrated to act as a central control point. The first insight was the requirement for a mitochondria-enriched fraction to enable cytosolic extracts to induce apoptosis in isolated *Xenopus eggs (*Newmeyer *et al* 1994). The elucidation of the pathway proceeded through the intense study of the activation of caspase-3 by the Apoptotic Protease Activating Factors (APAFs). A series of groundbreaking papers from Xiaodong Wang and colleagues used protein purifications of cell extracts to identify three key factors (APAFs) capable of activating caspase-3 in a cell free system, in the presence of dATP. In the first of the papers Liu *et al* (1996) purified a factor they termed APAF-2, which surprisingly turned out to be cytochrome-C. Normally present on the outer surface of the inner-mitochondrial membrane, cytochrome-C serves as a shuttle for electrons between complex III and complex IV of the mitochondrial electron transport chain (Brierley and Murer 1964). Subsequent work by two independent groups demonstrated the anti-apoptotic effect of Bcl-2 on the mitochondria was mediated by acting to prevent apoptosis induced cytochrome-C release, and hence prevent caspase activation (Kluck *et al* 1997; Yang *et al* 1997).

In the second of the papers Zou *et al* (1997) identified another factor, APAF-1, which on analysis was shown to be the first mammalian homologue of the *C. elegans* protein ced-4. The ced-4 domain of APAF-1 is flanked on one side by a region bearing strong homology to the prodomain of some caspases, CARD domains, and the other side by WD repeats, which were demonstrated to bind to cytochrome-C (Zou *et al* 1997). The final instalment by Li *et al* (1997) demonstrated that the third factor, APAF-3, was in fact identical to caspase-9. This led to the speculative model that cytochrome-C binding to the WD repeats of APAF-1 leads to a conformational change exposing its CARD domain and allowing caspase-9 to bind, resulting in its processing and activation (Li *et al* 1997; Reed 1997). This tripartite complex consisting of APAF-1, caspase-9, and cytochrome-C, in the presence of dATP, has been termed the "apoptosome" (Hengartner 1997). In retrospect, a better term could be the

"cytoplasmic apoptosome", since the death-induced signalling complex (DISC) involved in mediating death receptor signalling pathways could be considered a "membrane apoptosome", as both essentially represent a protein platform that serves to mediate procaspase aggregation and subsequent activation, coupled to downstream events (see below). A basic schematic of this pathway is outlined in Figure 1.2. In addition to cytochrome-C, other mediators of apoptosis are released from mitochondria. In some cells, mitochondria have been demonstrated to contain a pool of caspase-3 that is liberated into the cytosol on induction of apoptosis (Mancini *et al* 1998). Another recently described but phylogenetically ancient apoptotic mediator is the so-called apoptosis-inducing factor (AIF). On release of AIF from mitochondria it translocates to the nucleus where it mediates large scale (~50kb) nuclear fragmentation, and is thought to be involved in caspase-independent forms of cell death (Susin *et al* 1996; 1997).

1.4.5 Death receptors – the extrinsic pathway

In contrast to the intrinsic, extrinsic pathways represent a death decision that has originated from outside the cell, normally through the ligation of surface death receptors. Death receptors are members of the tumour necrosis factor (TNF) / nerve growth factor (NGF) superfamily, and members include TNF R1, Fas/Apo-1/CD95, DR-3/Apo-3/TRAMP, DR-4/TRAIL-R1, and DR-5/TRAIL-R2/TRICK2 (Schmitz *et al* 2000; Ashkenazi and Dixit 1998, and references therein). The common feature to all of these members is a characteristic cysteine-rich extracellular motif, and an important 68 amino acid homology region on their cytoplasmic tail named the death domain (DD) (Itoh and Nagata 1993; Tartaglia *et al* 1993). In short, death domain signalling proceeds through ligand-induced receptor trimerisation, recruitment of receptor associating adapter molecules, and subsequent initiation of a caspase cascade. Receptor ligation is thought to cause a conformational change of the cytoplasmic DD to allow homotypic interaction with DD containing adapter proteins such as FADD or TRADD, allowing their recruitment to the death induced signalling complex (DISC) (Chinnaiyan *et al* 1995). Fas death receptor signalling recruits FADD, whilst TNF R1 binds FADD through its initial interaction with TRADD. FADD is essential to both pathways due

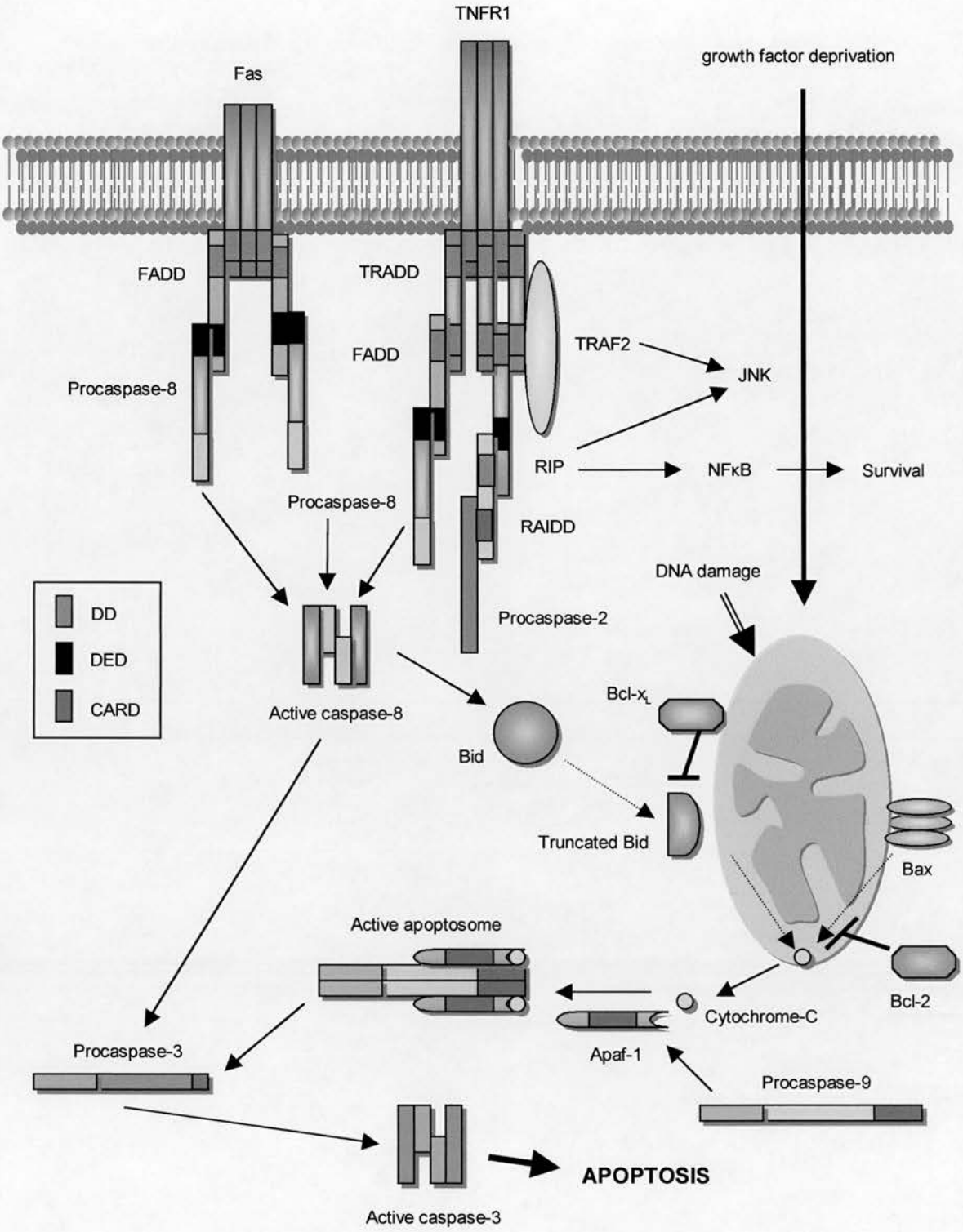


Figure 1.2: Basic schematic of the intrinsic and extrinsic apoptotic signalling pathways. Ligation of death receptors, induces processing of caspase-8 and subsequent activation of effector caspases – the extrinsic pathway. Intracellular changes in Bcl-2 family members mediate mitochondrial disruption and release of cytochrome-C, resulting in formation of the apoptosome and activation of caspase-3 – the intrinsic pathway. Caspase-8 cleavage of Bid induces release of cytochrome-C from the mitochondria, and as such represents a crossover of the pathways. Stress or chemical induced apoptosis also results in release of cytochrome-C. Adapted from Schulze-Osthoff *et al* (1998)

to it containing an N-terminal death effecter domain (DED), which allows interaction and hence recruitment of DED containing procaspases such as -8 and -10 to the DISC. As a result, procaspases are proteolytically cleaved into active forms enabling them to cleave downstream caspases (Srinivasula *et al* 1996; Muzio *et al* 1996; 1998). In addition, caspase-8 has been demonstrated to cleave the BH3 only protein Bid, resulting in its truncated form mediating release of cytochrome-C from the mitochondria (Luo *et al* 1998). A basic schematic of this pathway is outlined in Figure 1.2.

Controversy exists as to the relative requirement for cytochrome-C release in death receptor signalling. Whether a cell requires the "secondary amplification" of caspase activation that cytochrome-C release provides is thought to be dependent on cell type (Scaffidi *et al* 1998), and can be shown by the ability of Bcl-2 to inhibit death (Huang *et al* 2000). The TNF R1 pathway has also been shown to activate the TNF R1 associated factor 2 (TRAF2) through TRADD, which has been shown to be involved in NFκB activation-mediated survival (Ting *et al* 1996). In addition, receptor interacting protein kinase (RIP) has been shown to be able to mediate caspase-independent death of T cells following interaction with FADD after either Fas or TNF R1 ligation (Holler *et al* 2000). Regulation of death receptor pathways can occur by cell surface decoy receptors, such as DcR-1, -2, -3, all of which contain truncated cytoplasmic DD, and cannot mediate FADD or TRADD recruitment, hence acting as competitive inhibitors for ligands (Pan *et al* 1997; Marsters *et al* 1997; Pitti *et al* 1998). Control of death can also occur due the presence of endogenous FADD-like inhibitory proteins (FLIPs), which contain DED domains enabling them to interact and hence sequester other DED containing proteins such as procaspase-8, -10, and FADD (Hu *et al* 1997; Thome *et al* 1997; Tschopp *et al* 1998). Interestingly many viral immune evasion strategies involve manipulation of death receptor signalling. The viral protein crmA is an inhibitor specific for caspase-8, -10, and -1, thereby preventing the host defence strategy of killing the virally infected cell (Ray *et al* 1992; Komiyama *et al* 1994).

1.4.6 Regulation of caspases

Given the irreversibility of caspase activation by cleavage and the fundamental consequences that ensue, cells have developed many methods to prevent activation, antagonise their activity, and to promote their removal and destruction. The Inhibitor of Apoptosis (IAP) family of proteins have an evolutionarily conserved role in regulating apoptosis in species ranging from insects to humans (Clem and Miller 1994; Roy *et al* 1995; Rothe *et al* 1995). All IAPs contain one or more of three baculoviral inhibitory repeats (BIR), a characteristic cysteine-rich domain of ~80 amino acids (Rothe *et al* 1995). The extensively studied XIAP contains all three BIRs followed by a COOH-terminal RING zinc-finger, whilst the smallest, Survivin, contains a single BIR2 domain (Tamm *et al* 1998). Using an *in vitro* cell free system, XIAP was shown to prevent cytochrome-C induced caspase-3 activation and nuclear destruction. In addition, it could also inhibit active caspase-3 and the closely related caspase-7 activity by directly binding to them (Deveraux *et al* 1997). However, XIAP was demonstrated to bind weakly to their unprocessed forms, implying that its inhibitory effect on caspase-3 processing occurred upstream, namely by preventing activity of processed caspase-9 (Srinivasula *et al* 2001). Furthermore, it has been shown that XIAP itself can be cleaved by caspases into a BIR1-2 containing fragment, specific for caspase-3 and 7, and a BIR3-RING zinc-finger fragment, which specifically inhibits caspase-9 (Deveraux *et al* 1999). Interestingly, the introduction of mutations into XIAP, which abrogate its caspase-3 inhibitory activity, does not prevent it from protecting cells as efficiently as the wild type protein, suggesting its main target to be initiator caspases (Silke *et al* 2001).

Further investigations have shown the hunter becomes the hunted. XIAP itself has a panel of inhibitors, able to antagonise its anti-caspase activities by displacing and sequestering it. XIAP-associated factor 1 (XAF1) is a nuclear protein identified using the yeast two-hybrid system. It was shown to interact directly with XIAP, resulting in its sequestration in nuclear inclusions, reversing its protective effect (Liston *et al* 2001). Importantly, a screen of cancer cell lines found XAF1 to be absent or present at greatly reduced levels in most tested. In another key paper from Xiaodong Wang and colleagues, and simultaneously from David Vaux's group, a novel mitochondrial protein capable of antagonising XIAP was identified. Named either Smac (Du *et al* 2000) or Diablo (Verhagen *et al*

2000), it was demonstrated to be released from the mitochondria concomitant with cytochrome-C, and to directly bind XIAP through its BIR3 domain (Liu *et al* 2000). Given its release with cytochrome-C, its antagonism of XIAP further helps to amplify the caspase cascade.

Phosphorylation represents another important means by which caspases can be controlled. Ras activity in cells had been noted to confer resistance to apoptosis caused by stimuli known to release cytochrome–C (Yang *et al* 1997). Effectors of the Ras pathway include phosphatidyl-inositol 3-kinase, which generates secondary messengers that activate Akt, a serine-threonine protein kinase (Khwaja *et al* 1997). Treatment of Ras-transfected cell cytosols with protein phosphatases restored cytochrome-C mediated caspases activation. Pro-caspase-9 was shown to contain Akt consensus phosphorylation sites, which when phosphorylated at Ser^{196} resulted in conformational change of the catalytic machinery responsible for its auto-activation when complexed with APAF-1 and cytochrome-C (Cardone *et al* 1998). Another example of particular relevance given the context of this thesis is the inactivation of caspases by the calcium-activated neutral cysteine protease, calpain. Calcium has long been established as a regulator of apoptosis, and has been shown to promote or inhibit dependent on cell type. *In vitro* analysis of calpain activity has shown its cleavage specificities to be structure and not primary sequence-dependent (Sorimachi *et al* 1997). Several groups have demonstrated calpain to be able to cleave a number of caspases including -9, -8, -3, and -7. Processing occurs at sites that produce truncated forms, unable to activate downstream effectors or cleave substrates, effectively preventing most caspase cascades (Wolf *et al* 1999; Chua *et al* 2000; Lankiewicz *et al* 2000). This mechanism is particularly striking in excitotoxic neuron death, whereby over activation of glutamate receptors lead to neuronal calcium overloading. The apoptosis is initiated by cytochrome-C release, triggered by mitochondrial uptake of Ca^{2+}, but activation of calpains by the Ca^{2+} results in cleavage and inactivation of caspase-9 and -3, effectively converting neuronal death into a caspase-independent program (Lankiewicz *et al* 2000).

1.4.7 Caspase-independent cell death

That cells die by caspase-dependent apoptosis is universally accepted, but do all forms of cell death utilise caspases as their executioners? Recent evidence indicates a diversification of the apoptotic program in higher eukaryotes that questions the necessity and role of caspases, and in particular the many apoptotic-like cell death programs occurring without caspase activation (Lavoie *et al* 1998; Mathiasen *et al* 1999; Foghsgaard *et al* 2001). Surprisingly, many of these alterations to the death program do not ultimately affect the final removal of the dying cell (Chung *et al* 2000; Hirt *et al* 2000), and thus serve the same purpose as classical apoptosis – the safe non-inflammatory removal of corpses (Ren and Savill 1998). One could propose that the evolutionary pressure for an organism to develop several cell death programs reflects the increasing complexity and lifespan of the organism (Aravind *et al* 2001). It is well characterised that yeast undergo an apoptosis-like death associated with DNA fragmentation, zeiosis, PS exposure, and chromatin condensation, despite being devoid of caspases (Frohlich *et al* 2000). In addition, caspase coding sequences are conspicuously absent from the genomes of many non-animal species (Aravind *et al* 2001), including plants and single-celled eukaryotes, which have been demonstrated to undergo programmed cell death under conditions of stress (Ameisen 1996). The revelation of these "back-up" death pathways often occurs following pharmaceutical inhibition of caspases or genetic ablation, but several lines of evidence from both normal physiology and pathological conditions underscore the need for such a "second line defence". For example, caspase pathways can be inhibited by factors such as energy depletion (Leist *et al* 1997), nitrative/oxidative stress (Leist *et al* 1999), other proteases (Lankiewicz *et al* 2000; Chua *et al* 2000), the IAP family (Strasser *et al* 2000), and a range of viral proteins able to directly inhibit caspases (Strasser *et al* 2000).

A plethora of new terms have now been presented within the apoptosis field, helping to describe the many variations of cell death that do not match the strict morphological criteria of caspase-dependent apoptosis. These include; Apoptosis-like cell death, whereby chromatin condensation is less complete and compact, but recognition molecules for phagocytes are still displayed before cell lysis (Lavoie *et al* 1998; Mathiasen *et al* 1999; Foghsgaard *et al* 2001);

necrosis-like cell death, typically used to define cell death that occurs in the absence of any nuclear condensation, along with a range of accompanying apoptotic-like features including PS exposure (Vercammen *et al* 1998; Holler *et al* 2000) - a subset of this death type has been characterised as "aborted apoptosis", that is the initiation of a standard apoptotic program that is blocked at the level of caspase activation and is finally terminated by caspase-independent routes (Nicotera *et al* 1999) (Of particular relevance to chapter 4); paraptosis, characterised by little nuclear condensation, but extensive cytoplasmic vacuolisation (Wyllie and Golstein 2001), and many more obscure forms including autophagy (Xue *et al* 1999) and dark cell death (Turmaine *et al* 2000)!

1.5 Role of the Bcl-2 family and the Mitochondria

1.5.1 A family of pro- and anti-apoptotic proteins

The first gene found to be specifically involved in the process of physiological cell death in mammalians cells was bcl-2 (Vaux *et al* 1988). This proto-oncogene is located at the break point region of the t(14;18) chromosomal translocation commonly seen as a hallmark of follicular B cell lymphoma (Yang and Korsmeyer 1996). However instead of inducing cell proliferation, as with most other oncogenes at that time, it was unusually able to extend cell survival (Vaux *et al* 1988). An important discovery of molecular and functional similarity between Bcl-2 and the *C. elegans* gene product Ced-9 (Hengartner and Horvitz 1994) led to the finding that overexpression of Bcl-2 in ced-9 $^{-/-}$ nematodes could partially suppress death (Vaux *et al* 1992). Bcl-2 was found to be the seminal example of a continuously expanding family of apoptosis regulatory proteins, including Bcl-X_L, Bcl-X_S, Bcl-W, Bax, Bak, Bad, Bik, Bim, Bid, Mcl-1, A1, Hrk, Bok, Blk, Nip3, Nix and Diva, (Reed 1998; Adams and Cory 1998, and references therein) to name but a few! In addition viral homologues of Bcl-2 have been found in Adenovirus, Epstein-Barr virus, and African swine fever virus (Chiou *et al* 1994; Granville *et al* 1998; Meinl *et al* 1998), whereby inhibition of the cells' apoptotic response to the invading pathogen allows for a more productive and persistent infection. Strangely both pro- and anti-apoptotic regulators are encoded from the same homologous family of domains. All members contain a least one of four possible conserved motifs named the Bcl-2 homology domains (BHX)

(Adams and Cory 1998). The majority of pro-survival members include at least BH1 and BH2, with those closely related to Bcl-2 containing all four (Bcl-X_L, Bcl-W). Two subfamilies of the pro-apoptotic members contain either BH1 to 3 (Bax, Bak, Bok), or only the single short BH3 domain, and are referred to as the BH3-only proteins (Bid, Bad, Bik, Blk). Interestingly these BH3-only proteins share similarity to the *C. elegans* protein Egl-1, shown to be required for death (Conradt and Horvitz 1998). Curiously this suggests that many mammalian family members such as Bax or Bak have no homologue in the worm.

1.5.2 The duelling dimer hypothesis of death

Bcl-2 and Bax were shown to be able to form homodimers with themselves and to heterodimerise with each other (Oltvai *et al* 1993). This led to a model whereby the relative levels of Bcl-2 and Bax would determine whether anti-apoptotic Bcl-2 homodimers, Bcl-2-Bax heterodimers, or pro-apoptotic Bax homodimers formed (Oltavai and Korsmeyer 1994). Mutagenesis of the BH1-3 domains strongly implicated them in mediating dimerisation, and the crystal structure of Bcl-X_L provided an explanation. Coalescence of the α helices in its BH1-3 domains creates an elongated hydrophobic cleft into which a BH3 amphipathic α helix can bind (Muchmore *et al* 1996). This BH3 "cleft-coupling" may account for the ability of all family members to dimerise with each other, leading to the postulation that Bax and its analogues may have alternative conformations: a Bcl-X_L-like conformation, and another with the BH3 helix rotated outside to allow insertion into the groove of an anti-apoptotic family member. As well as the promiscuous interactions with themselves, Bcl-2 family members have been shown to interact with a range of proteins including APAF-1, cFLIP/Casper, Raf-1, R-Ras, calcineurin, and the adenine nucleotide translocator (ANT) of the mitochondrial inner membrane (Reed 1998). Evidence from the genetically tractable *C. elegans* model suggests a requirement for interactions between ced-9 and ced-4 (the APAF-1 homologue)(Ellis *et al* 1991). Physical interaction between ced-9 and ced-4 has been shown to correlate with the ability of ced-9 to suppress ced-4 mediated activation of caspases (Spector *et al* 1997; Wu *et al* 1997, Chinnaiyan *et al* 1997). Similarly, in the mammalian system the BH4 region of Bcl-X_L was shown to bind the apoptosome component APAF-1, interacting with the ced-4-like region

within this protein (Hu *et al* 1998; Pan *et al* 1998). However, although containing a BH4 region Bcl-2 itself binds APAF-1 very weakly *in vitro*, indicating the anti-apoptotic role of Bcl-2 in mammals is not APAF-1-directed, but mediated by some other mechanism.

1.5.3 Mechanisms of action of Bcl-2 family members

Although Bcl-2 family members have been extensively studied and are implicated in regulating mitochondrial cytochrome-C release, the precise biochemical foundation for this remains elusive. There are currently two basic models for possible mechanism, with substantial evidence for each. The Bcl-2 protein contains a stretch of hydrophobic amino acids at its COOH-terminus allowing insertion into intracellular membranes, primarily the cytoplasmic face of the mitochondrial outer membrane, endoplasmic reticulum, and the nuclear envelope (Krajewski *et al* 1993). However, despite containing COOH termini only a small fraction of Bcl-X_L is present on membranes, whilst Bax remains cytoplasmic until an apoptotic stimulus occurs (Hsu and Youle 1998). An insight into the mechanism of Bcl-2 family function arose from the realisation that the Bcl-X_L crystal structure, and in particular the α 5 and α 6 helices, bore a striking resemblance to pore-forming domains of some bacterial toxins, for example, diphtheria toxin and the colicins (Muchmore *et al* 1996; Satler *et al* 1997). As expected, Bcl-2, Bcl-X_L, and Bax all form ion channels when added to synthetic lipid bilayers, with those created by Bax and Bcl-2 displaying distinct characteristics including ion selectivity (Schendel *et al* 1997; Minn *et al* 1997; Antonsson *et al* 1997). Evidence for pore formation *in vivo* has not been demonstrated, and whether the pores formed are of a sufficient size to allow passage of cytochrome-C into the cytosol remains unclear. Other proteins released such as Smac/Diablo exists as a 100kDa end to end dimer (Chai *et al* 2000), suggesting a channel formed by ~20kDa proteins are simply not sufficient. The ability of Bax to oligomerise may partly explain this, with a report of a Bax tetramer able to form a channel in liposomes of around 22 Å, and therefore able to permit cytochrome-C passage (Saito *et al* 2000). However, small bacterial toxins such as pneumolysin can oligomerise to form a ring of 30-50 subunits and a channel size of 400 Å (Gilbert *et al* 1997), although this has yet to be shown with Bcl-2 family members.

A further proposed model for Bcl-2 family member modulation of cytochrome-C release may involve a more non-specific disruption of outer mitochondrial membrane. Following alterations in mitochondrial physiology, as a consequence of apoptotic signalling, a disruption may occur to allow diffusion of proteins across the lipid bilayer. This non-specific rupture is thought to require the opening of a large mitochondrial conductance channel termed the permeability transition pore (PTP), formed from a complex of the voltage dependent anion channel (VDAC), the adenine nucleotide translocator (ANT), and cyclophilin D (Zamzami *et al* 1996a; Qian *et al* 1997; Crompton 1999). Opening of the PTP allows small molecular weight solutes (<1.5kDa) to diffuse across the inner mitochondrial membrane resulting in a collapse of the inner mitochondrial membrane potential ($\Delta\psi M$) and matrix swelling (Zamzami *et al* 1996b). Swelling of the mitochondria is thought to result in rupture of the membrane, enabling release of cytochrome-C and other associated apoptogenic factors localised within the intermembrane space (Kroemer *et al* 1997b). It is suggested that Bcl-2 family members could directly regulate PTP activity to modulate factor release (Marzo *et al* 1998; Narita *et al* 1998). Supporting this model is the observation that inhibitors of PTP opening, such as bongkrekic acid and cyclosporin, are able to block apoptosis in some (Zamzami *et al* 1996a), but not all systems (Eskes *et al* 1998). Whether mitochondrial depolarisation occurs as a result of PTP opening to initiate apoptosis, or whether PTP opening occurs as a later event due to apoptotic changes remains highly controversial.

In another study VDAC alone has been demonstrated to interact with pro-apoptotic members of the family such as Bax and Bak, to allow formation of the so-called "megapore" (Shimizu *et al* 1999; 2000). Conversely anti-apoptotic family members such as Bcl-X_L may act to promote a closed conformation of the pore (Shimizu *et al* 1999). In many ways this "enlarged" VDAC model has more in common with the channel models discussed above. Studies of the BH3-only members, such as Bid, suggest they can act alone through a separate as yet undefined pathway, causing release of cytochrome-C. It is predicted Bid may associated with novel outer mitochondrial membrane proteins (Wang *et al* 1996; Luo *et al* 1998; Li *et al* 1998).

1.6 Hiding the evidence, removing the corpse

As mentioned in the historic background, the study of phagocytosis was originally reported in the late 19th century by Ilya Metchnikoff (1893). Using basic light microscopy he observed "microphages" (i.e. neutrophils) being "englobed" by macrophages in the experimentally injured fins of tadpoles, the fundamental biological phenomena we now know as phagocytosis – i.e. cells consuming other cells. The consumption of microorganisms and other foreign material (non-self) is an important primary host defence in innate immunity, whilst the phagocytosis of senescent or dead cells (altered-self) plays a pivotal role in embryogenesis, normal tissue turnover, development, regulation of the immune system, and in the resolution of inflammation (Hart *et al* 1996). The critical and central caveat of these processes is that dying or apoptotic cells are recognised and phagocytosed prior to them undergoing lysis. Hence avoiding the leakage of potentially deleterious intracellular components that would result in the damage of adjacent tissue. In short, phagocytic clearance is swift and efficient, leaving no remains of the apoptotic cell, and thus partly explaining why physiological cell death was overlooked for so many years. In fact, the classic quote is that on calculation "an entire tissue could disappear in 20 days with only four apoptotic cells being visible at any one time, in any given section" (Tidball and Albrecht 1998). Of course one could argue that this represents the phagocytes ability *in vivo* to perhaps recognise and clear dying cells before the classic hallmarks of apoptosis occur.

1.6.1 Phagocytes and their receptors

Phagocytes involved in the process of clearance can be considered to be either professional, such as macrophages, or semi-professional, such as epithelial cells, fibroblasts, vascular smooth muscle cells, and Sertoli cells (Chemes 1986). A number of receptors on the phagocyte surface have been implicated in the binding and uptake of apoptotic cells (Figure 1.3). These include lectin-like receptors that were suggested to interact with altered sidechains of surface glycoproteins on the apoptotic cell (Duvall *et al* 1985). The vitronectin receptor, an $\alpha_v\beta_3$ integrin thought previously to be

primarily involved in anchorage of cells, has been shown to be involved in the phagocytosis of a wide range of cells (Savill *et al* 1990). Extending this work, the class B scavenger receptor CD36 was also implicated in this process through interaction with thrombospondin, a glycoprotein synthesised and secreted by the macrophage. Thrombospondin is suggested to bind cooperatively with $\alpha_v\beta_3$ and CD36 to form a "molecular bridge" between phagocyte and apoptotic cell (Savill *et al* 1992). Other molecules implicated on the phagocyte include class A scavenger receptors (Platt *et al* 1996), the ATP-binding cassette transporter ABC1 (Luciani and Chimini 1996), activation of both classical and alternative complement pathways with subsequent coating of the apoptotic cell with C3bi, followed by recognition by macrophage complement receptors (Mevorach *et al* 1998), and CD14, the bacterial lipopolysaccharide (LPS) receptor (Devitt *et al* 1998). Interestingly, interaction between CD14 and its prototypical ligand LPS leads to an inflammatory response by the macrophage and subsequent release of proinflammatory cytokines (Pollack *et al* 1995), whereas uptake of apoptotic cells does not (Fadok *et al* 1998).

1.6.2 "Eat me" signals on the apoptotic cell

In comparison to the surface of the macrophage, much less is known about the molecules on the apoptotic cell that mediate clearance. In addition to the modification of glycoproteins mentioned above, the loss of phospholipid asymmetry and exposure of phosphatidylserine (PS) on the outer leaflet of the plasma membrane has emerged as an important marker of cell death and phagocytic clearance (Fadok *et al* 1992a; Martin *et al* 1995). Subsets of macrophages have been demonstrated to preferentially use PS as a signal for recognition and uptake (Fadok *et al* 1992b), however, the identity of the PS receptor on the phagocyte remained elusive for many years. Although many suggestions of candidate molecules had been made including annexins, CD36 (Tait and Smith 1999), and scavenger receptors (Rigotti *et al* 1995), no conclusive evidence demonstrated them to be the PS receptor. However, using a phage display screen for the antigen of mAb 217, known to block PS dependent phagocytosis, Fadok and colleagues (2000) successfully cloned a novel sequence with high homologies to proteins of unknown function in both *C. elegans*, and *D. melanogaster*. This putative PS-receptor specifically conferred the ability to phagocytose apoptotic cells on expression in Jurkat T

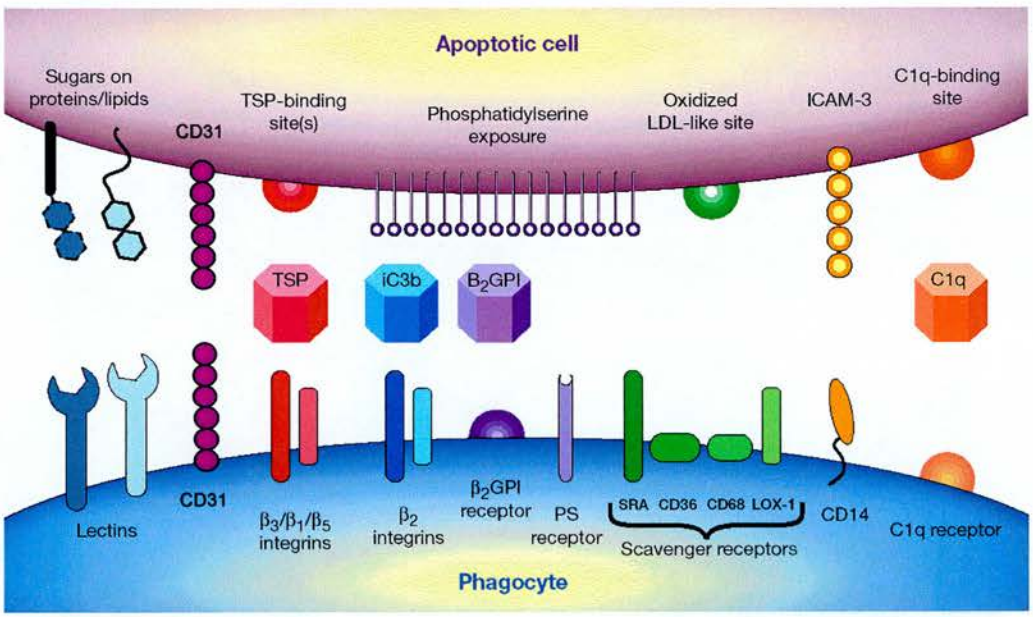


Figure 1.3: Schematic of the major molecular players involved in phagocytic clearance of apoptotic cells. Inhibitor studies in assays of the ingestion of apoptotic cells in vitro by phagocytes have revealed a variety of candidate molecules, many of which have incompletely understood roles or uncharacterized binding partners. A repertoire of 'eat me' signals (top) interact with receptors on the phagocyte (bottom), either directly or via serum-derived bridging molecules (middle). LDL, lowdensity lipoprotein; SRA, class A scavenger receptor; TSP, thrombospondin. Adapted from Savill and Fadok (2000).

cells, and displayed the characteristic downregulation of inflammatory function after uptake of apoptotic cells (Fadok *et al* 2000). Other molecules implicated on the dying cell's surface include the immunoglobulin superfamily member ICAM-3 (Moffat *et al* 1999), and the complement protein C1q (Botto *et al* 1998), however many of these proteins function are still yet to be fully understood.

1.6.3 The importance of being eaten

The apparent abundance and redundancy of phagocytic mechanisms is a keen indicator of the vital importance of phagocytosis *in vivo*. Acute inflammation, and in particular the participation of neutrophils, has evolved as a beneficial host response to infection and injury, and normally resolves with minimal damage to surrounding tissues (Ren and Savill 1998). However, the neutrophil is essentially a circulating "loaded weapon", with specialised granules full of lytic enzymes and inflammatory mediators. If these cells die by necrosis and release their contents at an already inflamed site, the potential to exacerbate local tissue damage is high (Savill *et al* 1993). The archetypal example is in the adult respiratory distress syndrome (ARDS), where a large influx of neutrophils into the lung results in the overwhelming of the clearance system, and subsequent secondary necrosis and tissue damage (Smith 1994). Therefore, the clearance of effete neutrophils must be considered an important prerequisite for the resolution of inflammation. In this respect work by Savill *et al* (1989) has provided evidence of the close link between the apoptotic fate of neutrophils and subsequent engulfment by macrophages *in situ* in the acutely inflamed joints of human patients.

1.6.4 It takes two to tango, the role of CD31

The "traditional" and universally accepted model of phagocytic clearance suggests that target cells undergo biochemical changes of apoptosis leading to the expression of "eat-me" signals on the surface, marking the cells and allowing engulfment. However, is this model "holistic" enough in an *in vivo* context? The observation that apoptotic cells are rarely seen within tissue sections has always been interpreted as a reflection of the efficacy of phagocytic clearance *in vivo*. Perhaps, however, the cellular changes able to mediate phagocytosis occur much earlier within the death program, and

before the relatively late classical markers of apoptosis, such as nuclear fragmentation and condensation. Given the deleterious nature of necrosis does it make sense for a cell to become recognition competent so late in the program? One could argue that a better physiological system would include some form of cross talk between predator and prey, with the death impending cell able to "communicate" its need to be cleared, and maybe even a timescale for this. In analogy, maybe a dying cell has a surface molecular equivalent of a "fuel gauge", able to be read by the phagocyte to indicate when the cell was likely to be "running on empty". Conversely, could a more viable cell be capable of registering its condition to the phagocyte, effectively telling it to "get lost"? Recent work from the group suggests that the relationship between predator and prey may, in some settings, be more intertwined than previously thought. Using a novel molecular "fishing" approach Brown and colleagues (2002) identified CD31, otherwise known as PECAM-1, to be able to mediate interactions between macrophages and neutrophils. Interestingly, fresh viable neutrophils were capable of mediating an active detachment from the macrophage, whilst apoptotic neutrophils could not, and hence were unable to escape and were subsequently phagocytosed.

Platelet structure and function

Platelets are small anucleate cells of discoid shape, measuring 1-3 μm in diameter. Formed in the bone marrow from the precursor cell, the megakaryocyte (MK)(Radley and Scurfield 1980), they are found almost exclusively in the circulation throughout their lifespan of ~8 days, and represent the most abundant blood cell after the erythrocyte, with normal levels ranging from 150-450,000 per μl of whole blood (Kumar and Clark 1998). Although not containing a nucleus, platelets are capable of a complex variety of reactions that are essential for haemostasis, vital for healing of damaged blood vessels, and play an as yet ill-understood part in inflammation. Platelets are extremely metabolically active utilising both glycolysis and oxidative phosphorylation to support their endocytic and secretory activities (Doery *et al* 1970). With virtually all their organelles, proteins and enzymes derived from the MK, platelets represent a unique "pre-packaged" powerhouse equipped with a full armoury of specialised factors, and a dedicated internal structure to enable their prime function, the maintenance of vascular integrity. Examination by TEM reveals platelets to be "jammed full" of granules that have been termed alpha and dense, dependent on their electron opacity (Figure 1.4). Alpha granules tend to contain factors involved in coagulation and adhesion, whilst dense granules tend to contain platelet agonists (Ginsberg *et al* 1980). In addition, many mitochondria are apparent throughout the cytoplasm, along with glycogen particles and smaller vesicles shown to contain lysosomal enzymes (Bentfeld-Barker and Bainton 1975). One of the most unique defining features of the platelet is the microtubule coil seen to encompass all internal organelles (Figure 1.4). This coil in conjunction with the actin cytoskeleton maintains the discoid shape. Mechanical or agonist-induced stimulation depolymerises the microtubules to cause shape change. Another distinctive function-specific platelet feature is the unusually thick glycocalyx, the "forest" of glycoproteins and receptors at the cell surface evidenced by fixing in the presence of ruthenium red, which aids the formation of fibrinogen and fibrin "bridges" between cells during aggregation (Savage and Ruggeri 1991).

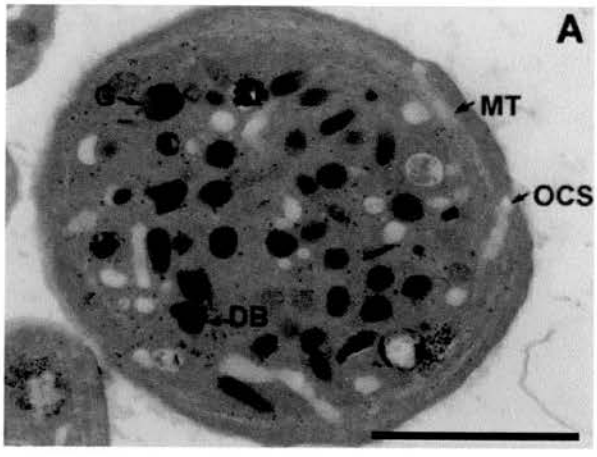


Figure 1.4: Transmission electron micrograph of a typical platelet. A typical cross section through an unactivated platelet showing a typical distribution of dense bodies (DB), alpha granules (G), microtubule coil (MT), and the open canalicular system (OCS). Scale bar represents 1 μm.

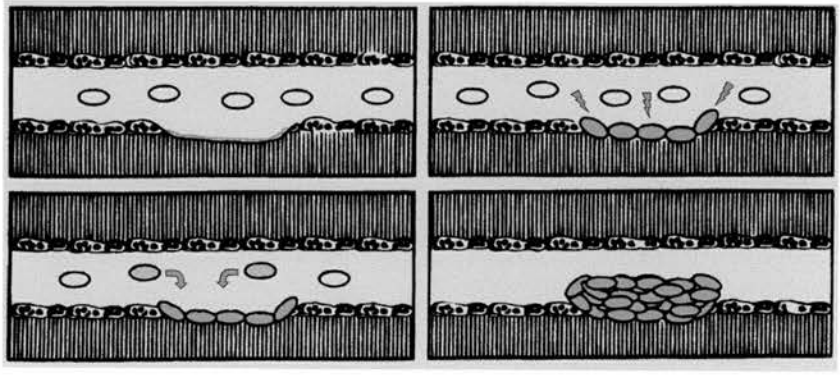


Figure 1.5: A basic schematic of platelets' haemostatic functions. On encountering exposed basement membrane, platelets adhere and release factors to recruit other platelets, forming a thrombus which acts as a physical plug.

1.7 Molecular basis of platelet adhesion

Resting platelets exist in the circulation as singular entities, and are thought to have little interaction with each other or other cell types. However, in response to appropriate stimuli they rapidly change to an adhesive state. A very simplified scenario of platelet adhesion to vessel wall is outlined in Figure 1.5. Damage to the endothelial cell lining of the vasculature, by for example the rupture of an atherosclerotic plaque, exposes underlying basement matrix proteins that support initial platelet attachment. Platelets flatten and spread (shape change) across the surface forming multiple tight interactions with the matrix and between each other. Spreading, in conjunction with other agonists released in the microenvironment, induce platelets to secrete granule contents causing additional resting platelets within the circulation to adhere, forming an aggregate that acts as an effective plug or seal to prevent excessive blood loss. Obviously this series of functional platelet responses, although essential for haemostasis, can be devastating if the platelet-rich thrombus formed occludes the blood vessel leading to thrombosis. Conversely, syndromes causing defects in these adhesive reactions, and usually of a genetically acquired origin (e.g., Glanzmann's thrombasthenia or Bernard-Soulier syndrome), can be equally as dangerous leading to latent bleeding episodes.

1.7.1 The endothelium and matrix proteins mediate platelet attachment and spreading

In an elegantly simple system the major components of the subendothelial matrix support platelet attachment and spreading (Table 1.2). With an intact endothelium creating an effective barrier, circulating platelets are kept away from the matrix preventing thrombus formation. On damage, these proteins become exposed initiating platelet attachment and spreading. In addition to creating an effective physical barrier, endothelial cells contribute to a non-thrombogenic surface by synthesising an elaborate array of molecules able to inhibit factors or directly effect aggregation and coagulation responses, notably nitric oxide (Radomski *et al* 1987), prostaglandin I_2, ecto-ADPase (CD39) (Marcus *et al* 1997), heparin sulphate proteoglycans (which are secreted directly into the subendothelium), thrombomodulin (Esmon 1995). However, damaged endothelium rapidly downregulates these anticoagulant functions to become procoagulant. Even in the absence of endothelial damage inflammatory mediators can tip the equilibrium to a pro-thrombogenic surface.

TNF and IL-1 have been shown to down-regulate thrombomodulin and induce expression of tissue factor on endothelial cells (Lijnen and Collen 1997), whilst endotoxin, TNF, and IL-1 increase the expression of the plasminogen activator inhibitor-1, impairing fibrinolysis (Maiser and Bulger 1996).

Matrix Constituent	Comment
Collagens	Family of proteins with type I, II, and IV supporting adhesion, spreading and secretion
von Willebrand factor	Multimeric protein critical for the hemostatic function of platelets
Fibronectin	Dimeric or multimeric protein which supports attachment and spreading of platelets
Thrombospondins	Trimeric proteins exhibiting adhesive and antiadhesive properties
Laminins	Proteins supporting platelet attachment
Microfibrils	A fibular bundle of protein constituents found in some matrices

Table 1.2: Subendothelial matrix components supporting platelet adhesion

However, a subset of platelet responses and an alteration of their attachment to the matrix constituents, listed in Table 1.2, occurs due to several compounding factors: Under some conditions platelets attach to, but do not spread on laminin (Ill *et al* 1984), whilst von Willebrand factor (vWF) and fibronectin support attachment and spreading (Sakariassen *et al* 1979). Collagen on the other hand not only supports attachment and spreading, but also mediates a secretory response (Brass *et al* 1974). A growing body of evidence has led to the suggestion that a phenotypic difference in endothelial cells of different blood vessels may alter the composition of the subendothelium, hence eliciting a different response (Ruf and Morgenstern 1995). Indeed one can envisage a tiny capillary may only warrant a "small layer" of platelets, and hence attachment and spreading are enough, whilst a major vessel would require the secondary secretory activities to amass a greater platelet plug. Vessel shear stress also substantially alters platelet adhesion, particular in reference to vWFs contribution. *In vitro* experiments have demonstrated a role for vWF-mediated attachment at high shear but not at low shear (Weiss *et al* 1978). Intriguingly, the major physiological stimulus for the continuous production of NO *in vivo* is shear stress, and hence areas of high shear, and hence high risk of platelet activation, bear a nonthrombogenic microenvironment (Schini-Kerth 1999). Fibronectin, however, supports attachment at high and low shear (Houdijk *et al* 1985). In addition, matrix proteins

can interact with one another. Thrombospondin, fibronectin, and vWF all bind to collagen, which may serve to bridge the platelet to the matrix protein (Santoro and Cowan 1982; Engvall *et al* 1978; Mumby *et al* 1984). Most matrix proteins are open to degradation by a range of proteolytic enzymes, which has been shown to alter the adhesive properties. In fact "cryptic adhesion sequences" have been elucidated, which on degradation and subsequent exposure mediate interaction with additional sets of platelet adhesion receptor (Pfaff *et al* 1993).

1.7.2 Platelet adhesion receptors and integrins

The individual matrix proteins discussed above interact with the platelet by acting as ligands for a panel of surface proteins, collectively termed adhesion receptors (Table 1.3). Multiple designations exist for each, with cluster of differentiation (CD) terminology, integrin family designations, and the original glycoprotein (Gp) nomenclature all regularly used. The oldest Gp term, and most favoured among platelet biologists, arose from the relative electrophoretic mobility of the proteins, whereby the slowest moving, and hence biggest molecular weight, was given the designation GpI, and so on. However, as electrophoresis became more advanced several proteins were found in the same positions and hence GpIa, GpIb, etc, were designated (Phillips and Agin 1977). In addition, several Gp members exist on the platelet membrane as non-covalent complexes, for example, GpIIb-IIIa, GpIc-IIa, and GpIb-IX, and as such are generally regarded as a single membrane protein. Although confusing in complexity, the inescapable fact is the redundancy that exists in the system, enabling a single matrix component to enact several distinct responses by interaction with different receptors.

As mentioned previously, many adhesive receptors are integrins. Integrins represent heterodimeric cell surface molecules that share structural, immunochemical, and functional homology (Hynes 1987). The α subunits, of which fourteen are known, are similar to one another, but the β subunits, of which eight are known, share a high degree of sequence homology of around 35-45% at the amino acid level. Each β subunit combines noncovalently with an α subunit to form an adhesive protein receptor. Platelets express two major β subunits, β_1 and β_3, and five α subunits, α_2 α_5 α_v α_{IIb} α_6

Ligand	Receptor(s)	Other designations
Collagen	GPIa-IIa	VLA-2, $\alpha_2\beta_1$
	GPIIb-IIIa	$\alpha_{IIb}\beta_3$, CD41/61
	GPIV	GPIIIb, CD36
	GPVI	
Fibrinogen	GPIIbIIIa	$\alpha_{IIb}\beta_3$, CD41/61
Fibronectin	GPIc-IIa	VLA-5, $\alpha_5\beta_1$
	GPIIb-IIIa	$\alpha_{IIb}\beta_3$, CD41/61
Thrombospondin	Vitronectin receptor	$\alpha_v\beta_3$, CD51/61
	GPIV	GPIIIb
	IAP	
Vitronectin	Vitronectin receptor	$\alpha_v\beta_3$, CD51/61
	GPIIb-IIIa	$\alpha_{IIb}\beta_3$, CD41/61
vWF	GPIb-IX	
	GPIIb-IIIa	$\alpha_{IIb}\beta_3$, CD41/61
Laminin	GPIc-IIa	VLA-6, $\alpha_6\beta_1$

Table 1.3: Platelet receptors for adhesion proteins.

(Kishimoto *et al* 1998). The integrin GpIIaIIIb ($\alpha_{IIb}\beta_{III}$) is one of the most narrowly distributed of blood cell integrins, appearing exclusively on platelets and MKs, and thus serving as a lineage marker. Integrins play an important role in platelet biology, and in particular during aggregation, due to an interesting transduction property termed "outside in" and "inside out" signalling.

1.8 Platelet secretory mechanisms

As mentioned in the preface to this section, once platelets have attached to exposed matrix they typically release a variety of substances capable of stimulating or inhibiting platelets and other blood cells, covalently modifying the thrombus to affect its mechanical properties, modulating the growth of cells within the vessel wall, and regulating coagulation. Platelets in effect can directly orchestrate the response at the site of injury, and mediate the longer term repair responses, much more than simply acting as a physical plug.

1.8.1 Mechanisms of secretion

The majority of platelet-derived substances are actively released from preformed storage granules, degranulation, or are directly synthesised at the membrane by modification to membrane phospholipids. Some factors also reside in the cytosol and are thought to be released through some degree of platelet lysis. Secretion by platelets is triggered by a variety of strong agonists, such as thrombin, binding and spreading on collagen, and only by weaker agonists such as ADP, in the presence of thromboxane A_2, when cells are brought into close proximity as during aggregation (Charo *et al* 1977). Incorporation of membrane markers into the alpha granules established exocytosis as the principal method of granule content release (Stenberg *et al* 1985; Wencel-Drake *et al* 1986; Berman *et al* 1986). However, different routes are taken by the alpha and dense granules, probably accounting for the relative difference in rate of release between the two. On stimulation dense granules appear to individually fuse with the plasma membrane (Morgenstern *et al* 1985), whilst alpha granules are seen to move toward the centre of the platelet, clearly not an ideal position to fuse with the plasma membrane (Ginsberg *et al* 1980; Stenberg *et al* 1984; Morgenstern *et al* 1987). One hypothesis proposes that the granules individually fuse with the deep invaginations of the plasma membrane running through the platelet, termed the open canalicular system (OCS), from where the secretory products are moved to the outside of the cell (White *et al* 1970). The other suggests that α-granules fuse with each other at the cell centre, forming a "compound granule". This compound granule then moves to the cell surface and fuses with the plasma membrane (Ginsberg *et al* 1980; Morgenstern *et al* 1987; Painter and Ginsberg 1984). However, there is also substantial evidence to support a great deal of inward membrane traffic, thought to act in clearing adhesive and procoagulant proteins from the cell surface (Wencel-Drake *et al* 1996), hence limiting thrombogenic events. In particular, internalisation of the GpIIbIIIa receptor complexed with fibrinogen occurs, and there appears to be a cycling intracellular receptor pool of GpIIbIIIa and GpIb (Wencel-Drake *et al* 1986; 1990; 1993).

1.8.2 Platelet granule contents and functions

The major platelet factors and their subcellular location are outlined in Table 1.4. The platelet membranes and the dense bodies produce a variety of short-lived mediators, either due to degradation or diffusion, and are the most rapidly secreted (Ginsberg *et al* 1980). Their contents have rapid effects on the cells of the local vessel wall and the behaviour of other platelets, in particular with regard to vessel tone. ADP acts as a potent agonist which recruits other platelets to the forming thrombus, whilst released ATP effects other blood cells, in particular causing Ca^{2+} fluxes in MΦ that inhibit phagocytosis (Sung *et al* 1985; Greenberg *et al* 1988). Platelets also represent the major blood source of the psychoactive serotonin, which again can alter vascular tone. The role of the divalent cations released is unclear, but is assumed to provide local excess for some of the calcium-dependent enzymes involved in coagulation and cross-linking of the thrombus. Thromboxane A_2, an arachidonate-derived mediator generated by hydrolysis of membrane phospholipids (Kroll and Schafer 1989), acts as a potent chemotractant to recruit other platelets, and has vasoconstrictive properties, thus reducing blood flow through the damaged vessel.

A wide variety of peptides able to modulate the gene expression and growth of the vessel wall cells are contained within the α-granules. The first to be investigated was the platelet-derived growth factor (PDGF)(Ross 1989), which occurs as three isoforms and has two known receptors occurring mainly on smooth muscle cells and fibroblasts (Heldin and Westeremark 1989). In normal physiology PDGF is thought to play a role in promoting wound healing after platelet interaction with a damaged vessel wall. However, it has also been strongly implicated in the overt proliferation of the intimal smooth muscle cells after interaction with atherosclerotic plaques (Ross 1979). Transforming growth factor-β (TGF-β) was first isolated from platelets, which represent an abundant source of this cytokine. Along with having inhibitory effects on macrophage activation, TGF-β has complex effects on cell proliferation, inhibiting in some systems whilst promoting in others. Again platelets represent the major blood source of Thrombospondin, a large ~450kDa protein that is known to play a role in platelet aggregation, angiogenesis (Dawson *et al* 1997), phagocytosis (Savill *et al* 1992), and in the activation of TGF-β (Majack *et al* 1988).

Dense body	Cytoplasm	Alpha-granules		
Agonists	Cytoplasmic factors	Adhesive proteins	Growth modulators	Coagulation factors
ADP	Factor XIII	Fibrinogen	PDGF	Factor V
ATP	PDECGF	Fibronectin	CTAP III	HMWK
Serotonin		vWF	TGF-β	C1 INH
Calcium		Thrombospondin	Platelet factor 4	Fibrinogen
		Vitronectin	Thrombospondin	Factor XI
				Protein S
				PAI-1

Table 1.4: Substances released from platelets and their intracellular location. PDECGF, platelet-derived endothelial cell growth factor. VWF, von Willebrand Factor. PDGF, platelet-derived growth factor. CTAP III, connective-tissue-activating peptide III. TGF-β, transforming growth factor β. HMWK, high molecular weight kininogen. C1 INH, C1-esterase inhibitor. PAI-1, plasminogen activator inhibitor-1.

Many of the platelet α-granule contents are proteins found within the plasma, however whether platelets directly scavenge these or if MKs biosynthesise, for example fibrinogen, remains controversial. Although present within the blood a wide variety of coagulation factors reside within platelets. Platelets contain large amounts of factor-V, which plays a role in the assembly of prothrombinase, one of the final steps of coagulation (Mann *et al* 1981). The plasminogen activator inhibitor-1 (PAI-1), an inhibitor of urokinase and tissue plasminogen activators, is also released in high concentrations from the α-granules (Erickson *et al* 1984). It is tempting to speculate that to complement the blood derived factors, platelets hold a cache of important "rate limiting" factors of direct benefit in thrombus formation at higher microenvironment concentrations, factor-V catalysing thrombin generation, and PAI-1 in preventing immediate fibrinolytic activities in the vicinity of the newly formed thrombi (Braaten *et al* 1993; Fay *et al* 1994). The α-granule protein P-selectin (CD62P, PADGEM, GpIIa, GMP140) represents an unusual case. It resides on the membrane of the α-granules but on activation is rapidly expressed on the cell surface, occurring even before degranulation is apparent (Sternberg *et al* 1985). As mentioned previously α-granules centralise and coalesce before release, and the OCS does not become incorporated into the plasma membrane, as in other specialised secretory cells such as basophils and mast cells (Zucker-Franklin 1989). Explanations have suggested

that P-selectin is translocated by lateral flow within the plane of the membrane (White and Escolar 1990), but given that P-selectin can be detected within seconds of activation these seem unlikely.

1.9 Molecular basis of platelet aggregation

To summarise, platelets maintain haemostasis in three main ways: by sticking to collagen and then to one another (aggregation) at sites of vascular injury they form part of the physical plug that prevents blood loss; by accelerating the rate at which coagulation proteins are activated, and hence the rate of fibrin deposition to strengthen the clot; and by secreting granule contents to cause local vasoconstriction, recruit more platelets, and promote eventual wound healing. The central caveat of this process is how a free circulating platelet changes into a sticky interacting platelet. Broadly

Agonist	Comment
ADP	Acts synergistically with many other agonists
Thrombin	Formed by activation of the coagulation system
Collagen	Constituent of subendothelial matrix
Epinephrine	May allow hormonal control of haemostasis
Calcium ionophore	Synthetic drug; mobilises calcium within platelets
Arachidonate metabolites	Formed and released from plasma membrane of activated platelets
Serotonin	May act primarily to sensitise platelets to other agonists
Platelet-activating factor	Lipid mediator produced by, and able to activate other cells and platelets

Table 1.5: Common platelet activating agents

speaking platelets need a stimulus to initiate this range of responses, of which the key factors are listed in Table 1.5. Of particular physiological significance are the platelet derived agonists ADP, serotonin, and the arachidonate metabolites, as mentioned previously, all of importance in recruiting and activating other platelets. The key protease thrombin, which is the most powerful platelet agonist *in vivo*, connects the blood coagulation system with thrombus formation, cleaving the soluble fibrinogen into the insoluble fibrin.

1.9.1 Molecular requirements for platelet aggregation

Platelet aggregation is evidently different from agglutination, induced by ristocetin or certain platelet antibodies, by its energy requirement. In addition, three main factors are required for true aggregation: platelet agonist, divalent cations, and fibrinogen. With, for example, ADP treatment only, platelets undergo a shape change from a discoid to a more spherical form bearing pseudopodia, reflecting rearrangement of the underlying actin cytoskeleton, but aggregation will not occur until fibrinogen and Ca^{2+} is added. The requirement for agonist, fibrinogen, and Ca^{2+} together is easily understandable once the mechanism of the platelets interaction with fibrinogens is described. Resting platelets bind little fibrinogen, however on stimulation and in the presence of Ca^{2+} around 50,000 molecules of fibrinogen are seen to interact with the cell (Bennett and Vilaire 1979; Marguerie *et al* 1979; Mustard *et al* 1979), an amount comparable to the number of GpIIbIIIa receptors on the platelet surface. As fibrinogen is a symmetric molecule it is able to bind two GpIIbIIIa receptors and hence two platelets. Thus, when repeated 50,000 times a very tight union between a mass of platelets is formed - the aggregate. The competence to bind fibrinogen occurs within seconds of platelet stimulation, indicating a receptor conformational change and not the presentation of a new receptor on the surface. The key event is the Ca^{2+}-dependent conversion of the GpIIbIIIa receptor from a low affinity to a high affinity state able to bind fibrinogen. The reversible phase of fibrinogen binding occurs with a K_d of around 0.3μm (Bennett and Vilaire 1979; Marguerie *et al* 1979; Marguerie and Plow 1981) that may appear low, but given the plasma concentration of fibrinogen (10μm)(Kumar and Clark 1998) it represents a thirty fold molar excess. During this reversible phase chelation of divalent cations causes dissociation of fibrinogen from GpIIbIIIa, and hence dissolution of the aggregate. However, by an unknown mechanism a time-dependent stabilisation occurs leading to irreversible fibrinogen binding, correlating to irreversible platelet aggregation, and removal of divalent cations is no longer effective (Marguerie and Plow 1981; Muller *et al* 1993). The stability given by irreversible binding may help maintain the thrombus in a microenvironment that progresses to conditions that limit platelet activation and thrombus growth.

1.9.2 The fibrinogen receptor – GpIIbIIIa, integrin $\alpha_{IIb}\beta_3$

The key to platelet aggregation and pharmacological intervention for therapeutic benefit is the mechanism underlying the conformational change from fibrinogen binding incompetent to competent. Occurring as the most abundant cell surface protein on platelets and representing around 15% of surface protein, GpIIbIIIa is also contained within α-granules, with platelet activation able to double the copy number (Gerrard *et al* 1980). GpIIb, the α_{IIb} subunit, is synthesised in the MK as a single polypeptide chain, but is proteolytically cleaved to a heavy and light chain held by a disulphide bond (Duperray *et al* 1989), whilst the GpIIIa is the β_3 subunit and is known to complex with the α_v subunit to form the vitronectin receptor on platelets and other cells (Ginsberg *et al* 1987). The two subunits interact non-covalently in a 1:1 ratio soon after biosynthesis, and interaction is required for efficient surface expression (Jennings and Phillips 1982; O'Toole *et al* 1989; Duperray *et al* 1989). Stabilisation of the complex requires μM concentrations of Ca^{2+} (Kunicki *et al* 1985; Brass *et al* 1985; Steiner *et al* 1991), whereas conformational change to fibrinogen binding requires a mM concentration.

The process whereby agonist stimulated platelets internally transmit signals to change the GPIIbIIIa conformation has been referred to as "inside-out" signalling (Shattil *et al* 1994; Clark and Brugge 1995). Many aspects of this activation mechanism have been extensively studied, but the biochemical basis for the final transition is still not known. Many classes of signal response elements become activated following agonist stimulation, and mutation studies have definitively shown the GpIIbIIIa cytoplasmic tail to modulate affinity states (Shattil *et al* 1994; Shattil and Ginsberg 1997). Proteins found to interact with the cytoplasmic tails include the cytoskeletal components α-actinin, paxillin, talin, and filamin (Schaller *et al* 1995; Horwitz *et al* 1986), focal adhesion kinase (Schaller *et al* 1995), integrin-linked kinase-1 (Hannigan *et al* 1996), and the potential regulatory proteins beta3-endonexin, cytohesin-1, integrin cytoplasmic domain associated protein-1, and calreticulin (Shattil *et al* 1995; Kolanus *et al* 1996; Chang *et al* 1997; Coppolino *et al* 1995). Binding of ligand (fibrinogen) to the active integrin induces a further conformational change in GpIIbIIIa, exposing regions termed ligand-induced binding sites (LIBS) (Frelinger *et al* 1988; 1991). This secondary conformational

change results in a series of intracellular changes including activation of serine/threonine kinases and phosphatases, a process conversely called "outside-in" signalling (Schwartz *et al* 1995; Shattil *et al* 1994). Whether this secondary change alters, or is required for aggregation processes is not known, but some evidence has shown instances of fibrinogen bound to active GpIIbIIIa without induction of aggregation (Schwartz *et al* 1995).

1.10 Platelet clearance and cell death

1.10.1 Platelet cell death

All cells must die. Therefore even the anucleate platelet, with a defined half-life *in vivo*, will at some stage lose function and need to be "marked" to allow removal from the circulation. For the majority of mammalian cells, initiation of the apoptotic cell death program results in rapid morphological and biochemical changes that ultimately result in cell surface changes able to mediate their swift recognition and engulfment by phagocytes. However, relatively little data can be found on any form of cell death program within the platelet, with most investigations having looked at the parallels between platelet activation and apoptosis, such as PS externalisation, spectrin cleavage, and fragmentation into membrane bound vesicles. An early study by Vanags *et al* (1997) examined changes in members of the Bcl-2 family following ionomycin stimulation. By RT PCR platelets were confirmed to contain mRNA for both Bax and Bcl-2, which shifted to a more proapoptotic ratio on ionomycin stimulation, demonstrating platelets to be capable of altering the balance of these important mediators of cell death. In addition, agonist induced PS exposure by platelets was found to be independent of caspases or calpains, whilst caspase-3 activity remained at basal level following stimulation (Vanags *et al* 1997). Conversely, a second study identified caspase-3 as a key participant in platelet activation, being found processed to its active form, and responsible for PS exposure, microvesicle release, and cleavage of moesin (Shcherbina and Remold-O'Donnell 1999). However, the study was badly controlled, used inappropriate protease inhibitor concentrations, and inferred active caspase-3 from loss of proform alone. The caspase-independent nature of apoptosis-like PS exposure was strengthened by a third study in which calpain was demonstrated to be responsible for

PS exposure, microvesiculation, and gelsolin cleavage in activated platelets, and the processing to inactive forms of recombinant caspase-3 and -9 (Wolf *et al* 1999).

Two *in vivo* studies from the same group suggest older populations of platelets within dogs express PS on their surface, and undergo mitochondrial alterations. Using an estradiol-mediated model of thrombopoiesis suppression, it was found that during platelet decline, and hence increasing mean age of those remaining in the circulation, the percentage of PS positive platelets significantly increased (Pereira *et al* 1999). Using an identical model in the second study, changes in platelet $\Delta\psi M$ were studied using potential sensitive dyes. Again as older platelets appeared a loss of $\Delta\psi M$ was apparent, concordant with PS exposure (Pereira *et al* 2002), however no study of caspase activity was undertaken (perhaps their third paper!). From all this we can see that no real study of any constitutive and potentially age-induced death program within platelets has ever been made.

1.10.2 Platelet clearance

Considering around 200 billion platelets are removed and destroyed ever day, surprisingly little work has been conducted on the molecular mechanisms mediating this. Hundreds of radioactive tracer experiments have proposed the spleen and liver to be the primary and secondary site of platelet removal, respectively, as evidenced by the large numbers localising to these sites (Kaplan and Saba 1978). However, given the known sequestration of platelets and other blood cells by these two organs, whether this truly reflects clearance remains unknown. In fact the central dogma of most platelet clearance experiments to date has been the assumption that if they are no longer circulating then they must have been cleared. Given that many of the experiments have involved protease or chemical treatments to alter surface molecules (Greenberg *et al* 1975; 1979), it is quite possible that the adhesive properties of platelets have been increased, given the appearance of them having been cleared. To our knowledge no TEM has ever been published of a platelet that has been internalised or phagocytosed in an *in vitro* or a physiological *in vivo* context.

Much early work focussed on the role of platelet sialic acid on the platelet surface, and demonstrated that its removal with the sialidase neuraminidase caused an apparent clearance of these platelets by the liver within an hour of reinfusion (Greenberg *et al* 1975; 1979). Given the more recent understanding that sialic acid removal exposes previously hidden "siglec" binding sites, able to mediate cell-cell interactions (Munday *et al* 1999), these findings may be somewhat misguided. Platelet aggregates or thrombi are phagocytosed in part by monocytes and neutrophils (Zucker-Franklin 1989), therefore many groups have investigated the role of platelet activation markers in mediating clearance, and in particular P-selectin. Fluorescent-labelled thrombin activated platelets exposing P-selectin that are reinfused back into baboons are found to lose P-selectin surface expression, are not cleared from the circulation when compared to untreated controls, and do not lose haemostatic functions (Michelson *et al* 1996). In support of this, comparison of platelets from wild type and P-selectin deficient mice show an identical half-life of 4.7 days, and again activation of these platelets did not alter life span (Berger *et al* 1998). The aetiology of idiopathic thrombocytopenia purpura, an autoimmune disease causing low circulating platelet numbers, is thought to occur through immune complex mediated platelet clearance (Goad *et al* 1994; Cordiano 1996). Human platelets express FcγRIIA as their sole Fc receptor (Karas *et al* 1982; Cassel *et al* 1993). As mice lack the genetic equivalent for FcγRIIA, transgenic mice were generated to express FcγRIIA on both platelets and macrophages. Treatment of the mice with anti-mouse platelet antibody resulted in severe thrombocytopenia compared to control littermates due to immune mediated clearance, occurring even in the absence of the FcR γ-chain subunit (McKenzie *et al* 1999). However, the half-life remained the same between control and transgenic mice, suggesting FcγRIIA does not mediate clearance of aged platelets (McKenzie *et al* 1999).

Megakaryocyte structure and function

Not only is the megakaryocyte (MK) the most conspicuous and largest cell within mammalian bone marrow, it is arguably the most fascinating and unique. With the relatively recent cloning and subsequent commercial availability of the MK differentiation and growth factor thrombopoietin (TPO), researchers finally have the ability to expand MKs *in vitro*, and these elusive cells are slowly beginning to give up their secrets. It is a polyploid cell (not to be confused with polynucleated), which refers to the multiple duplications of the chromosomes from the usual 2N up to 64N, all contained within the same nuclear envelope. Multiple rounds of abortive mitosis, which has been termed endomitosis, result in a large multilobed nucleus and a cell body up to 60μm in size. The multiple endomitotic events almost certainly serve to increase the volume of the cell, akin to normal mitosis but without the cell division, enabling MKs to undergo their prime function, the production of large numbers of platelets. Following final maturation and biosynthesis of platelet specific organelles the MK extends long processes containing platelet-sized nodes, termed proplatelets, from the tips of which individual platelets are released. Each mature MK is capable of releasing hundreds of platelets, with daily production balancing destruction at around 2×10^{11} platelets (Harker and Finch 1969).

1.11 Megakaryocyte development

1.11.1 Megakaryocytopoiesis

MK development proceeds within the bone marrow from the pluripotent myeloid stem cell, and is more easily understood when divided into three general stages: progenitor cells, immature MKs or promegakaryoblasts (PMkB), and mature MKs. As in erythropoiesis, progenitor cells are responsible for expansion of committed MK numbers, and can be induced to proliferate in response to a number of general mitotic growth factors such as stem cell factor and IL-3, and by the lineage specific factor thrombopoietin (TPO). PMkBs are transitional, spanning the mitotic progenitors to the mature post-mitotic MKs. The mature MKs do not proliferate, but do continue DNA synthesis

through endomitosis. *In vitro* studies have revealed the earliest detectable progenitor committed to the lineage to be the MK high-proliferative-potential colony-forming cell (MK-HPP-CFC), whose prolific abilities lead to macroscopically visible colonies of a few thousand MKs (Long *et al* 1988; 1989; Lowry *et al* 1995). The burst-forming unit MK (BFU-MK) is more mature and thought to be the direct progeny of the MK-HPP-CFC (Long *et al* 1989), but still leads to the classical "bursts" of individual colonies of around 100-500 MKs (Long *et al* 1985). The CFU-MK is the most differentiated of the progenitors with a limited proliferation potential of 2-32 MKs, and with a very particular cytokine requirement. Such is the requirement that the first CFU-MK colony was not reported until 13 years after Donald Metcalf's (1966) original bone marrow cultures (Fauser and Messner 1979; Vainchenker *et al* 1979).

The PMkB are transitional cells and are not readily recognisable by morphology, however the expression of MK/platelet specific markers such as GpIIbIIIa, and vWF begins to occur (Rabellino *et al* 1979; 1981). They lack proliferative ability, but continue DNA synthesis by endomitosis. *In vitro* they respond to a wide variety of haemopoietic growth factors, such as IL-3, c-kit ligand, IL-6, and TPO, maturing into single large MKs (Vainchenker *et al* 1982; Long *et al* 1982). PMkB are highly sensitive to TPO, and as such are the first observable cells to increase in number after induction of thrombocytopenia, and to decrease under conditions of thrombocytosis (Jackson 1973; Long *et al* 1979). The mature MKs are the first morphologically recognisable, and have been divided into three stages reflecting this: stage I typically bears a high nucleus to cytoplasm ratio; stage II tend to show great increases in total cell volume and granule content; whilst stage III/IV "granular MKs" are the most mature platelet producing cells. In general the lineage-specific antigen expression, granule content, and DNA content increases with this maturation.

1.11.2 Cytokine regulation of megakaryocytopoiesis

The regulation of MK numbers can be divided into three areas: the expansion of progenitor cells, the regulation of the maturation of these progenitors, and the control of terminal maturation and platelet formation. Extensive work has revealed a number of cytokines able to modulate the first two,

however to date, with the exception of extreme pharmacological intervention, little conclusive evidence has been described on the physiological regulation of platelet shedding. However, evidence suggests the role of the ECM components and stromal cells surrounding MKs *in vivo* may significantly contribute to the effect of the cytokine milieu. Although the MK growth and differentiation factor TPO has been confirmed to have powerful proliferative and maturational effects through all stages of development (Zeigler *et al* 1994; Young *et al* 1996; Sitnicka *et al* 1996), optimal *in vitro* expansion has shown to require factors such as stem cell factor (SCF), IL-3, IL-6, and IL-11 (Broudy *et al* 1995; Ku *et al* 1996). However, much of the work remains controversial with widely differing results between rodents and humans, between *in vitro* and *in vivo*, and a belief that many of the pleiotropic cytokines such as IL-6 may be regulators under stress conditions such as inflammation (Norol *et al* 1998). In addition, the cytokine driven proliferation of contaminating non-MK cells may have profound inhibitory effects on MK maturation (in particular IL-3 mediated myeloid lineage growth) (Lazzari *et al* 2000). The preferred consensus appears to be that TPO induces the differentiation of MK-committed haemopoietic progenitor cells, whereas IL-3, SCF, IL-6, and IL-11 trigger extensive growth of progenitors, which are then "channelled" by TPO into MK differentiation pathways (LaIuppa *et al* 1997; Lazzari *et al* 2000). Whatever the true nature of the complex cytokine interactions, TPO is very much established and accepted as the definitive megakaryocytopoiesis factor, able alone to drive MKs into full terminal differentiation and platelet production.

1.11.3 Thrombopoietin

TPO had been postulated to exist over forty years ago, and had been proved in principle as an activity within the plasma of thrombocytopenic animals (low platelet counts) that could stimulate platelet production on transfer to a second recipient (Odell *et al* 1961; Spector 1961; Schulman *et al* 1965). The search for TPO continued unsuccessfully for many decades, but in the early nineties Vignon *et al* (1992; 1993) cloned murine and human homologues of the v-*mpl* oncogene that is transduced by the myeloproliferative leukaemia virus. The c-*mpl* gene encoded a protein with strong homology to the haemopoietin receptor superfamily, and was found expressed on cells of haemopoietic origin. Further RT-PCR studies demonstrated c-*mpl* to be present on platelets MKs,

and CD34+ cells, and that specific antisense oligonucleotides inhibited MK colony formation *in vitro* (Methia *et al* 1993). The data established this receptor as important in megakaryocytopoiesis, and a role for the still unidentified c-*mpl* ligand. As always in research, the definitive cloning of TPO along with associated work to demonstrate its effects *in vivo* occurred shortly afterwards by no less than five independent groups (Lok *et al*; de Sauvage *et al*; Kaushansky *et al*; Wendling *et al*; Bartley *et al*, all 1994). The TPO gene encodes a protein with an expected size of 36kDa,which is heavily glycosylated to around 65-85kDa (Gurney *et al* 1995). Containing two domains, TPO has an amino terminal bearing strong homology to the erythrocyte differentiation factor erythropoietin, whilst the COOH-terminal contains serine and proline rich stretches (Lok *et al* 1994). Promoter regions of the gene show binding sites for transcriptional activators including GATA-1, NF-E2, and Ets family members (Deveaux *et al* 1996).

The system regulating circulating TPO levels, and hence MK numbers, is so elegantly simple and effective it reassures one's belief in evolution and its ability to develop the simplest answers to complex problems. Countless studies over the last 30 years have shown an inverse relationship between circulating platelet mass and TPO (or TPO-like activity pre-1994). The data fitted the idea that the main feedback mechanisms regulating circulating TPO levels was its binding to c-*mpl* positive cells, i.e. platelets, MKs and CD34+ cells. Under most conditions the production of TPO by the liver or kidney occurs constitutively and at a static basal rate, with mRNA levels not altered on changes in platelet numbers (Fielder *et al* 1997; Cohen-Solal *et al* 1996). Therefore, during periods of thrombostasis platelet mass remains constant and TPO remains at a basal level. However, during thrombocytopenia platelet mass has dropped and hence less TPO is "mopped up" resulting in a higher TPO concentration and the ensuing MK proliferation. Conversely, during thrombocytosis or primary thrombocythaemia (high platelet counts), platelet mass has increased resulting in lower circulating TPO and the concomitant reduction in MK proliferation and maturation. (Fielder *et al* 1996; Kuter and Rosenberg 1995).

Many of the signal transduction pathways activated by the binding of TPO to its receptor c-*mpl* have been elucidated. Intracellular domains of c-*mpl* closest to the transmembrane region are

required to induce the proliferative response, and have been shown to activate the Janus kinases / signal transducers and activators of transcription (JAK / STAT) signal transduction pathway (Gurney *et al* 1995; Morella *et al* 1995). TPO binding activates JAK2, which phosphorylates the transcription factors STAT 1, 3, and 5, with STAT 5 thought to be the major component (Drachman and Kaushansky 1995; Sattler *et al* 1995). In addition, ligand binding results in phosphorylation of the receptor itself (Sattler *et al* 1995), along with activation of PI-3 kinase, and phospholipase-Cγ (Drachman and Kaushansky 1997).

1.12 Platelet formation by megakaryocytes

1.12.1 Mechanics of platelet formation

The fact that platelets originate from MKs following terminal maturation is universally accepted (Wright 1906; 1910; Thiery and Bessis 1956a; 1956b), however the process by which this shedding occurs has remained elusive and dogged by controversy. Elucidation of the process has been hampered by the relatively low abundance of MKs within the bone marrow, ~0.05% of cells, and by the lack of reliable culture systems resulting in terminal maturation. These constraints resulted in most original work being conducted by TEM examination of fixed MKs, and as such forced the development of the original "demarcation model" of platelet shedding (Yamada 1957; Zucker-Franklin and Petursson 1984). Mature MKs are replete with platelet-specific granules, and contain unique internal membrane structures termed demarcation membranes (DMs). The essence of the model is that these DMs outline (demarcate!) future platelet territories, and hence platelets exist as nascent preformed entities with all specific organelles present within the mature MKs. It is then proposed MKs leave the bone marrow intact and under go circulation shear-induced fragmentation at the first capillary bed they encounter, usually the lungs (Trowbridge *et al* 1982). Examination of MKs by TEM certainly reveals platelet-sized fields between DMs, and since granules are plentiful within the MKs cytoplasm these fields appear as "platelets". However, this model oversimplifies platelets, tending to view their genesis as a problem of packaging granules into membrane-bound "bags", and thus overlooking their rich and unique cytoskeletal architecture. To this extent, immunofluorescence

microscopy, immunogold TEM, and SEM analysis of mature MKs have all failed to detect the characteristic microtubule coil or platelet-specific antigens within or along the DM platelet fields (Choi *et al* 1995; Italiano *et al* 1999)

The second model proposes that mature MKs extend long filamentous extensions, termed proplatelets, containing many platelet-sized nodes along their length, much like the appearance of a pearl necklace (Becker and DeBruyn 1976; Radley and Scurfield 1980). This model is not mutually exclusive of the previous as it makes two major reinterpretations: platelets are assembled de novo within the extended proplatelets; and that the DMs are not a partition, but serve as a reservoir for the vast quantity of plasma membrane required to package platelets (Radley and Haller 1982). Although numerous groups have contributed to the overwhelming evidence for the proplatelet model, by far the most convincing is the time-lapse phase microscopy by Italiano *et al* (1999) of a mature MK extending proplatelets and eventually releasing mature platelets (Figure 1.6). In combination with immunofluorescence microscopy and analysis by SEM of isolated cytoskeletons, they were able to correlate proplatelet morphogenesis with distinct ultrastructural changes to the cytoskeleton. Initial extensions of large pseudopodia were shown to contain unique parallel bundles of microtubules that elongated to form thin proplatelets with bulbous ends. These ends were shown to contain a peripheral bundle of microtubules that appeared to loop back onto itself to form a teardrop shaped structure. The importance of microtubules in driving the formation was revealed by the use of nocodazole, which blocked proplatelet extension, and taxol, which resulted in the formation of one or two very large abnormal processes. The timelapse also revealed proplatelets to be dynamic structures, undergoing multiple bending and bifurcation events to amplify greatly the number of tips from a single extension. Interestingly, treatment with cytochalasin B resulted in multiple long processes, devoid of bends or bifurcations implicating an actin-dependency. Use of an anti-tubulin Ab revealed microtubule coils to exist only at the very distal tips of the processes, and not within the platelet-sized nodes. This suggests that final formation and release of "mature" platelets only occurs at these tips, explaining the need for the multiple bifurcations of proplatelets as a means of amplifying platelet release. This model is somewhat validated *in vivo* by mice lacking the transcription factor NF-E2. MKs derived from these mice undergo a late arrest in maturation, resulting in a severe thrombocytopenia.

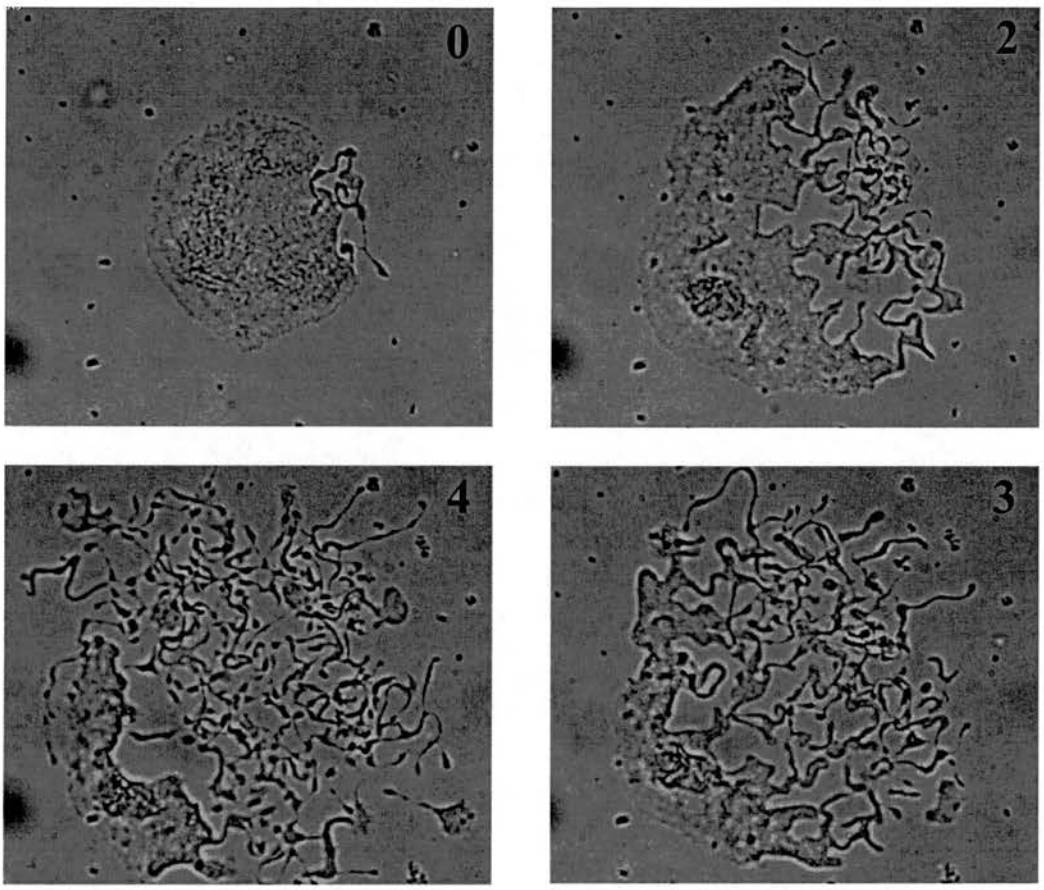


Figure 1.6: A mature megakaryocyte forming platelets. Phase time-lapse video microscopy of a mature murine MK extending proplatelets and eventually shedding functional blood platelets. Time points shown are 0 h, 2h, 3h, 4h. Pictures adapted from Italiano *et al* (1999).

In contrast to normal murine MKs that develop many proplatelets, MKs derived from the *NF-E2* $^{-/-}$ mice never produce proplatelets (Lecine *et al* 1998), underscoring the model as the physiological mechanism of platelet release.

1.12.2 Control of platelet formation

Although the basic mechanics of platelet production are becoming clearer, details of how the event is initiated and controlled remains vague and contradictory. Culture of whole human bone marrow cultures with serum results in little proplatelet formation, whilst serum-free conditions result in many proplatelets (Norol *et al* 1998). This data lead to the conclusion that serum must be inhibitory, ignoring the plethora of papers detailing that other bone marrow cells, kept proliferating by the serum, inhibit MK maturation. TPO has been reported to both promote (Nagahisa *et al* 1996) and inhibit (Choi *et al* 1996) proplatelet formation, and its ability to increase platelet numbers *in vivo* is almost certainly indirect via increases in MK mass. In contrast, plasma has been reported to promote proplatelet formation (Choi *et al* 1995; Leven and Yee 1987), as has coating culture vessels with extracellular matrix. Interestingly, matrix components reported to promote proplatelet formation include vitronectin (Leven and Tablin 1992; Leven 1995) and glycosaminoglycan-serglycin (Hunt *et al* 1993), both of which are found in the perivascular area of the bone marrow, exactly where platelet formation has been proposed to occur (Becker and DeBruyn 1976; Scurfield and Radley 1981). A pharmacological inhibitor based strategy investigated the effect of various cell signalling pathways. Proplatelet formation was found to be unaffected by inhibitors of PI-3 kinase, ERK, p38 MAP kinase, and protein kinase-A signalling pathways. However, blockade of protein kinase-C (PKC) with bis-indolylmalemide I, or down regulation by prolonged exposure to phorbol myristate acetate resulted in a significant inhibition of proplatelet formation. Use of a PKC class specific ribozyme strategy revealed the alpha isoform to specifically be involved (Rojnuckarin and Kaushansky 2001), whilst novel PKCα antisense oligonucleotides under clinical trials for the treatment of cancer has revealed the major *in vivo* side effect to be thrombocytopenia (Nemunaitis *et al* 1999; Yuen *et al* 1999).

Using a seven-step protein purification strategy a Japanese group claim to have found a putative proplatelet formation factor, isolated from normal human plasma (Ishida *et al* 2001). Based on its amino acid sequence the protein was identified as the protease inhibitor antithrombin III (AT III), known to be involved in the coagulation cascade. Interestingly the factor was found within the HDL fraction of plasma, which was shown to be required for its activity. Addition of thrombin to the AT III and HDL fraction to form the thrombin / antithrombin III complex (TAT) resulted in a two fold increase in the factor's efficacy, producing more than a ten fold increase in proplatelet extension above control. Intriguingly, the TAT complex is formed to prevent thrombin from acting during the "resolution phase" of coagulation. Therefore, its postulated that following acute platelet loss through bleeding and platelet consumption the activated coagulation cascade will result in the production of TAT, allowing a rapid stimulation of platelet release (Ishida *et al* 2001). Although the report is yet to be validated, reproduced, or extended by any other group, the ideas presented are of great interest.

1.12.3 Megakaryocytes and apoptosis

The concept that MKs can undergo apoptosis is well established with multiple reports of MKs with apoptotic morphology to be found within bone marrow. The MKs appear as condensed nuclei with a small veil of cytoplasm surrounding, indicative of having undergone platelet shedding, and have been described as denuded MKs (DMKs) (Radley and Haller 1983). In addition MΦ have often been sighted in the vicinity of these DMKs with processes extended to partially or completely engulf the dying cell (Radley and Haller 1983). An *in vitro* study demonstrated MKs grown out in culture for over 18 days underwent apoptosis constitutively to become TUNEL positive, propidium iodide positive, and to display nuclear fragmentation and a highly granulated cytoplasm. Although the study had evidence of some apoptotic cells they clearly had a very high amount of necrotic cells, "masquerading" within their quantifications as apoptotic (Zauli *et al* 1997). An interesting two part study by Battinelli *et al* (2000; 2001) studied the effect of nitric oxide (NO) on the induction of apoptosis in the MEG-01 cell line, concluding that NO exerted a powerful pro-apoptotic stimulus. Interestingly, they demonstrated that TPO was able to suppress this NO-mediated death. This effect of TPO to be able to suppress apoptosis of MKs had in fact previously been suggested to be a

mechanism allowing them to continue endomitosis and increase cell volume (Borge *et al* 1997). The very recent second part of the study proposed NO-induced apoptosis could induce MEG-01 cells to produce platelets (Batinelli *et al* 2001). Unfortunately their assay for platelet numbers was based on size of GPIIbIIIa positive particles as assayed by forward scatter (FSC) by flow cytometry, and made no real functional or morphological study of the particles produced. In addition, proplatelet bearing MK morphology was not alluded to or shown, and SEM micrographs revealed a MK morphology, that they proposed to be responsible for their platelet formation, indistinguishable to that reported for ROCK I mediated apoptotic membrane blebbing (Coleman *et al* 2001; Sebbagh *et al* 2001). Despite the many weaknesses of these studies they have set precedence for the possibility that MK apoptosis is associated with platelet formation, but do not conclusively show whether it is a cause or an effect.

1.13 Aims

The initial aims of this study were to try and elucidate whether human blood platelets undergo a form of constitutive cellular death, and subsequently whether this program could account for their safe clearance by phagocytes.

Preliminary studies investigated changes platelets had undergone during extended culture, including alterations in members of the Bcl-2 family, assessment of function, examination of subcellular changes by electron microscopy, and cell surface changes. In addition, aged platelets were assayed for their specific recognition and clearance by phagocytes. Subsequent experiments tried to elucidate molecular mechanisms responsible for the apparent "intrinsic death" of platelets. Involvement of the ubiquitous mediators of cell death, the caspases, were investigated. Furthermore, upstream mitochondrial changes, release of cytochrome-C, and apoptosome formation was assessed.

Finally, work was undertaken to establish whether caspases were involved in initiating or driving the birth of platelets from their progenitor cell, the megakaryocyte, and established whether a classical Fas death could induce platelet formation. In addition, the mechanistic contradiction of "life from death" was considered, investigating how a caspase death could be "compartmentalised" to allow formation of viable platelets capable of oxidative metabolism throughout their own lifespan.

Chapter 2 – Materials and Methods

2.1 Materials

All chemicals were of analytical reagent grade and purchased from **Sigma Chemical Co (Poole, UK)**, and all tissue culture media and supplements were obtained from **Gibco Life Technologies (Paisley, UK)** unless stated otherwise. Animals were obtained from the Department of Biomedical Resources, University of Edinburgh. Further materials were purchased from the following companies:

Adobe Systems Incorporate, San Jose, CA, USA: Photoshop 6.0

Alexis Corporation UK Ltd, Nottingham, UK: Fas ligand and enhancer

Amersham Pharmacia Biotech UK Ltd, Buckinghamshire, UK: Hybond-P polyvinylidene fluoride (PVDF) membrane, ECL plus detection reagent, Dextran T500, Percoll. 2D-E equipment; MultiPhor II 2D-E unit, Immobiline DryStrip IPG gels, IPG buffer, IPG cover fluid.

Ancell Corporation, Bayport, MN, USA: CD62P blocking mAb clone G1/G1-4

Anthos Labtec Instruments, Salzburg, Germany: Automatic plate reader

ATCC, Manassas, VN, USA: MEG-01 megakaryoblastic cell line

Bachem AG UK Ltd, St. Helens, UK: zVAD-fmk, zDEVD-fmk, zLEHD-fmk

Baxter Healthcare Ltd, Glasgow, UK: Diff Quick stain, saline solution 0.9% (sterile)

Becton Dickinson, Cowley, UK: FACScalibre flow cytometer

Biodata Corporation, Horsham, PA, USA: PAP4 aggregometer

BioSource International, Camarillo, CA, USA: Anti-human Bid pAb

Boehringer Mannheim, Mannheim, Germany: Annexin-V-FLUOS

Calbiochem-Novabiochem UK, Nottingham, UK: Caspase-3 substrate (DEVD-AMC), ALLN

Caltag Laboratories UK Ltd, Towcester, UK: CD61-FITC pAb

Carl Zeiss Ltd, Welwyn Garden City, UK: Axiovert S100 inverted microscope

Cecil Instruments, Cambridge, UK: Spectrophotometer CE2021

Chemicon International UK Ltd, Southampton, UK: Human recombinant caspase-9

CoolSnap, Leiden, The Netherlands: Air cooled CCD camera

Costar UK Ltd, High Wycombe, UK: Tissue culture plates, flasks, pipettes

Dako UK Ltd, Cambridgeshire, UK: Horse radish peroxidase (HRP)-conjugated goat anti-rabbit secondary pAb

Falcon, Becton Dickinson, Cowley, UK: Various plastic labwear; centrifuge tubes, pipettes, multiwell tissue culture plates

Improvision, Coventry, UK: OpenLab 3.0 image acquisition and analysis software

Intergen, Oxford, UK: CaspaTag fluorescent active caspase reagent

Intracel, Royston, UK: Willco glass bottomed microwell dishes

Leica Microsystems AG, Heidelberg, Germany: TCSNT confocal microscope system

Martindale Pharmaceuticals Ltd, Romford, UK: Calcium chloride

Molecular Dynamics, Amersham Pharmacia Biotech UK Ltd, Buckinghamshire, UK: STORM 860 phosphoimager, ImageQuant acquisition and analysis software

Molecular Probes Inc, Leiden, The Netherlands: JC-1 [5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazocarbocyaniniodide]; FLUO-3, Alexa Fluor 488 goat anti-mouse secondary Ab

Ortho Diagnostics, no longer trading: PermeaFix permeabilisation and fixation reagent

PeproTech EC Ltd, London, UK: Human thrombopoietin (TPO), human IL-1β

Perkin Elmer, EG&G, Wellesley, MA, USA: Spectrofluorimeter LS50B, FL WinLab acquisition software

Pharmingen, Becton Dickinson, Cowley, UK: Caspase-3 pAb, caspase-9 mAb (clone B40), caspase-9 pAb, APAF-1 pAb

Philips Electron Optics, Eindhoven, The Netherlands: CM150 transmission electron microscope (TEM)

Phoenix Pharmaceuticals Ltd, Gloucestershire, UK: Sodium citrate solution (3.8%)

Pierce Biotechnology Inc, Rockford, IL, USA: BCA protein assay

Polysciences Europe Gmbh, Eppelheim, Germany: 10 μm polystyrene beads

R&D Systems Europe Ltd, Oxon, UK: Human TNFα

Serotec Ltd, Kidlington, UK: CD41-PE pAb

StemCell Technologies, London, UK: StemSpan H2000 serum replacement media

Upstate UK Ltd, Botolph Clayton, UK: Human anti-Fas mAb clone CH-11

Zetos, EOBM, Philipps-University, Marburg, Germany: Zetos bone culture and loading system

The following reagents were kindly donated as gifts: Megakaryoblastic cell line SET-2 was kindly provided by K.Uozumi, Faculty of Medicine, Kagoshima University, Japan.

2.2 General Buffers

General cell lysis buffer with protease inhibitors

10 ml Hepes 100 mM pH 7.4
10 ml EDTA 10mM
1 ml Benzamidine 100mM
138µl Pepstatin A 5mg ml^{-1}
1 ml σ-PA 19.8 mg ml^{-1} (in MeOH)
86 µl Leupeptin 5 mg ml^{-1}
120µl Antipain 5 mg ml^{-1}
1 ml PMSF 17.4 mg ml^{-1} (made up fresh in MeOH)
To 100 ml with ddH_2O

1x Laemmli sample buffer

0.8 ml Tris pH 6.8
1 ml SDS 20%
1 ml Glycerol
0.53 ml β-Mercaptoethanol
~5 mg Bromophenol blue
To 10 ml with ddH_20

3x Laemmli sample buffer

2.4 ml Tris 1 M pH 6.8
3 ml SDS 20%
3 ml Glycerol
1.6 ml β-Mercaptoethanol
~5 mg Bromophenol blue
To 10 ml with ddH_20

Sealing gel solution

0.5 ml 30% Acrylamide
2 ml ddH_2O
20 µl APS (100mg ml^{-1})
10 µl TEMED

Separating gel solutions

	6%	**9%**	**12%**	**15%**
30% Acrylamide	2 ml	3 ml	4 ml	5 ml
Tris 2 M pH 8.8	2 ml	2 ml	2 ml	2 ml
ddH_2O	6 ml	5 ml	4 ml	3 ml
10% SDS	100 µl	100 µl	100 µl	100 µl
APS (100mg ml^{-1})	50 µl	50 µl	50 µl	50 µl
TEMED	25 µl	25 µl	25 µl	25 µl

Stacking gel solution

3.6 ml ddH_2O
0.7 ml 30% Acrylamide solution
0.6 ml Tris 0.5 M pH 6.8
50 µl 10% SDS

25 µl APS (100mg ml^{-1})
5µl TEMED

Anode running buffer (bottom tank)

30 g Tris
5 g SDS
5 L ddH_2O
pH to 8.6

Cathode running buffer (top tank)

144 g Glycine
30 g Tris
5 g SDS
5 L ddH_2O
pH to 8.6

Transfer buffer

144 g Glycine
30 g Tris
10 L ddH_2O
pH to 8.6

Blocking buffers

5 g non-fat dried milk powder
100 ml PBS
± 100 µl TWEEN-20
or
2 g Casein
100 ml PBS
± 100 µl TWEEN-20

Wash buffer

500 ml PBS
± 500 µl TWEEN-20

Developer buffer x10

6.1 g Tris
29.2 g NaCl
500 ml ddH_2O
pH to 8.0

Nuclear lysis buffer

10 ml Tris 100mM pH 8.0
20 ml NaCl 2M
20 ml EDTA 10 mM
To 100 ml ddH_2O
pH to 8.0

2.3 Cell culture

2.3.1 Maintenance of cell lines

Human megakaryoblastic cell line MEG-01 (Ogura *et al* 1985) (ATCC) and Jurkat T cells (Jurkats) were routinely maintained in RPMI 1640 supplemented with 10% FCS, L-glutamine (2 mM), and penicillin/streptomycin (0.1 mg ml^{-1}). Human megakaryoblastic cell line SET-2 (Uozumi *et al* 2000) was maintained in DMEM supplemented with 10% FCS, L-glutamine (2 mM), penicillin/streptomycin (0.1 mg ml^{-1}), 2-ME (10 μM), and 10% NEAA. Jurkats were cultured in suspension at a density of between 0.4 -1 $x10^6$ ml^{-1}, splitting every 2-3 days. Megakaryoblastic cell lines were similarly cultured in suspension, but at a density of between 0.1 - 0.5 $x10^6$ ml^{-1}, and only required splitting every 5 days.

2.3.2 Isolation of human mononuclear cells

Human neutrophils were purified from the peripheral blood of healthy human volunteers by modification of previously described methods (Haslett *et al* 1985; Dransfield *et al* 1994). Neutrophil isolation was performed at room temperature, under sterile conditions and using endotoxin-free reagents and plasticware (Falcon). Venous blood was collected into 50 ml polypropylene tubes, anticoagulated (4 ml 3.8% sodium citrate/36 ml blood) and centrifuged (350g, 20 min., RT), to give two layers. The upper layer contained plasma and platelets and a lower layer contained a mixture of erythrocytes and leukocytes. Platelet-rich plasma (PRP) was aspirated and used to prepare autologous serum in glass tubes by the addition of $CaCl_2$ (220 μl of 1 M $CaCl_2$ added to 10 ml PRP) at 37°C. To sediment the erythrocytes, 5 ml of 6% dextran (T500; pre-warmed to 37°C) was added to the pelleted cells and the volume made up to 50 ml with 0.9 % saline (pre-warmed to 37°C). The tubes were mixed gently and the cells allowed to sediment at RT (~30 min), resulting in formation of two distinguishable layers; a bottom layer containing mainly sedimented erythrocytes and an upper leukocyte-rich layer. The leukocyte-rich layer was aspirated, centrifuged (350g, 6 min) and the supernatant discarded. The resulting leukocyte pellet was resuspended in 2.5ml of 55% isotonic

Percoll (9:1 v/v Percoll:10 x PBS) in 1 x PBS w/o. Discontinuous Percoll gradients were prepared by overlaying 2.5ml of 68% Percoll onto 2.5ml of 79% isotonic Percoll in a 15 ml Falcon tube. Leukocytes were then resuspended in 55% Percoll and overlayed to form the final layer of the gradient. The gradients were centrifuged (720g, 20 min) and polymorphonuclear cells harvested from the 68% / 79% Percoll interface. Mononuclear cells sedimented at the 55% / 68% Percoll interface. Purified cells were washed sequentially in PBS twice and cell yield assessed using a haemocytometer. Cell viability was assessed by trypan blue exclusion and was routinely >99%. The typical yield for this isolation method was 100 x 10^6 polymorphonuclear and 50 x 10^6 mononuclear cells/40 ml whole blood.

2.3.3 Generation of primary monocyte derived macrophages

Freshly purified human mononuclear cells were plated at 4 x10^6 ml^{-1} in Iscove's Dulbecco's modified Eagles medium and allowed to settle and attach (~1 h). Non-adherent cells were washed off with two washes of HBSS w/o, followed by the addition of Iscove's containing 10% autologous serum (PRPDS), and incubated at 37°C for 6-7 days. Detached lymphocytes were removed by washing on day 2, and 5, replacing media with fresh Iscoves's 10% autologous serum.

2.3.4 Generation of primary MKs

Generation of primary murine megakaryocytes is described in detail elsewhere [16,17] (Drachman *et al* 1997; Rojnuckarin and Kaushansky 2001). Briefly, bone marrow was flushed from femurs of 6-8 week old male BALB/c mice and a single cell suspension obtained by gentle pipetting. Cells were incubated overnight in StemSpan H2000 (StemCell Technologies) with human recombinant TPO (40 ng ml^{-1}; PeproTech) and all non-adherent cells transferred to fresh wells and incubated for a further 72 h. Enrichment of mature megakaryocytes was by velocity sedimentation through a discontinuous 1.5%, 3.0% BSA gradient, retaining those cells reaching the bottom within 30 min. Purified primary MKs were replated and cultured with TPO (30 ng ml^{-1}) and 10% normal human plasma. Staining of purified MKs for CD41 (1:1000; Serotec) and CD61 (1:1000; Caltag

Laboratories) followed by FACS analysis revealed >95% to be highly positive for both markers, indicative of mature MKs.

2.3.5 Treatment of primary MKs and MK cell lines

Primary or cell line MKs were treated with reagents as indicated as follows; zVAD-fmk (100 μM; Bachem), zDEVD-fmk (10 μM; Bachem), zLEHD-fmk (10 μM; Bachem), Calpeptin (100μM; Calbiochem), anti-Fas agonistic antibody CH.11 (50 ng ml^{-1}; Upstate), anti-Fas antagonistic antibody ZB4 (1 μg ml^{-1}; Upstate), soluble Fas ligand and enhancer (5 ng ml^{-1}; Alexis), TNF-α (25 ng ml^{-1}; R&D Systems), IL-1β (25 ng ml^{-1}, Peprotech), and JO-2 (250 ng ml^{-1}; Pharmingen).

2.3.6 Platelet isolation and culture

Freshly drawn venous blood was obtained from aspirin-free healthy donors, citrated (0.33 %; Pharma Hameln), and PRP prepared by centrifugation (350 g, 20 min). PPP was prepared from PRP by further centrifugation (1200g for 15 min). Washed platelets were prepared by diluting PRP with 5 volumes of HBSS w/o, pH 6.4, containing EDTA (4 mM final) into a round-bottom capped polystyrene centrifuge tube before centrifugation (280 g, 20 min). After washing platelets were resuspended in HBSS w/o, pH 6.4, or media as indicated, and maintained at 37 °C in a closed round bottom polystyrene tube at 3 x 10^8 ml^{-1}. Platelet samples for SDS-PAGE were purified free of the majority of contaminating leukocytes by three successive centrifugations of the PRP / HBSS / EDTA (220 g, 5 min), retaining the supernatant each time, before finally pelleting the platelets by centrifugation (280 g, 20 min). Platelet preparations were routinely checked by microscopy for the presence of leukocytes or aggregates which if found resulted in the experiment being discarded.

2.3.7 Platelet activation

For analysis of activated platelets by TEM, fresh washed platelets resuspended in HBSS w/o, pH 6.4, were treated with ADP (10 μm) or Thrombin (3 U ml^{-1}) at 37°C (1 min) before fixation with 2.5% glutaraldehyde as detailed below. For positive controls of CD62P or PS exposure, 5 μl of fresh PRP was added to 500 μl of PBS and activated as above before staining and sampling by flow cytometry.

2.3.8 Phagocytic recognition of aged platelets

For experiments involving phagocytic recognition of aged platelets, following isolation platelets were first incubated in HBSS w/o Ca^{2+} at 5 x10^9 ml^{-1} (10 min) with Cell Tracker Orange (1.8 μM; Molecular Probes). Unincorporated dye was removed with two washes of HBSS w/o, pH 6.4, before resuspending at 3 x10^8 ml^{-1} in HBSS w/o, pH 6.4, and aging in culture. Platelets labelled with Cell Tracker Orange were washed free of conditioned media and resuspended in HBSS w/o before addition to a prewashed monolayer of adherent phagocytes cultured in 24-well plates. Typically 5 x 10^7 platelets were incubated with 1 x 10^5 phagocytes at 37°C for 10 min for platelets aged in the absence of plasma and 30 min for platelets aged in citrated plasma. Following the incubation period, the phagocyte monolayer was washed free of non-interacting platelets and any adherent platelets removed by treatment with trypsin at 37°C (5 min), followed by 5 mM EDTA at 4°C (20 min), to recover the human Mϕ, and trypsin/EDTA treatment alone at 37°C (5 min) for all other cell lines tested, before flow cytometric, confocal, and epifluorescence microscopic analysis. For phagocyte inhibition experiments MDM monolayers were preincubated at 37°C (10 min) with a panel of inhibitors diluted in Iscoves w/o serum at concentrations as indicated. Monolayers were then rewashed and the target platelets added to the wells along with inhibitors.

2.3.9 Treatment of platelets with proteases and a sialidase

Aged washed platelets in HBSS w/o (1 ml) were incubated at 37°C (30 min) with the proteases Elastase, Protease 9, Protease 14, Chymotrypsin, or Papain (all 10 μg ml^{-1}), or the sialidase Neuraminidase (0.01 U ml^{-1}). Following incubation platelets were washed free of proteases by centrifugation (280g, 5 min), resuspended in HBSS w/o to the original concentration, and transferred to a prewashed phagocyte monolayer, before incubation and analysis of phagocytosis as detailed above.

2.4 *Ex-vivo* bone culture

Trabecular bone from femoral heads was isolated from a 58-year old male patient undergoing hip surgery, machined with high precision to cylindrical cores under sterile conditions, and inserted into a Zetos™ bone perfusion and loading chambers (Smith and Jones 2001). Each core was individually perfused with 5 ml DMEM under recirculation at a rate of 5 ml h^{-1}, maintained at 37°C / 5% CO_2, and subjected to a daily 5 Hz loading cycle which was found necessary to ensure viability. A brief schematic of the system is outlined in Figure 2.1. On day 5, anti-Fas antibody CH.11 (50 ng ml^{-1}) and/or zVAD-fmk (100 μM) were added directly to reservoirs containing fresh media that was perfused to the bone cores. Perfused media was collected over an 18 h period, after which the flow rate was increased to 15 ml h^{-1} for 2 h, with a final flush of the core with 5 ml of PBS. Large cellular material was removed by centrifugation (200 g, 5 min) and remaining supernatant components concentrated by further centrifugation (1500 g, 15 min). Resuspended pellets were stained for GpIIb-PE (1:1000; Serotec) and GpIIIa-FITC (1:1000; Caltag), and analysed on a FACScan (Becton Dickinson), with reference to a fixed count of 10 μm beads (Polysciences) for platelet enumeration.

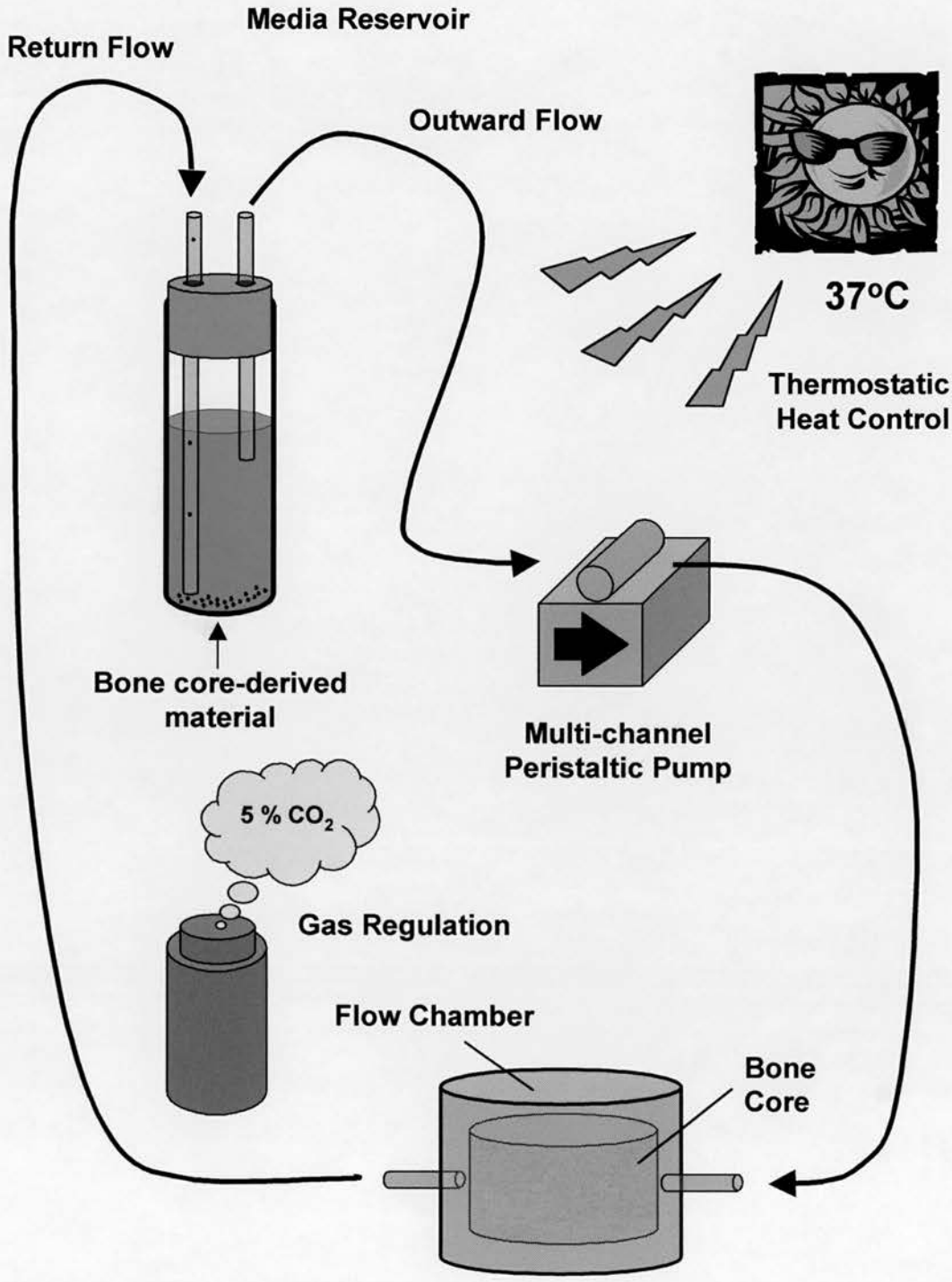


Figure 2.1: Schematic of the Zetos™ *ex-vivo* bone core culture system: Human trabecular bone, typically obtained during hip surgery, is high precision-machined under sterile conditions to multiple cylindrical cores and inserted into flow chambers. Up to 24 flow chambers and cores are connected to a looped media perfusion system driven by a multi-channel peristaltic pump. Each core has an individual media reservoir that is pre-gassed with 5% CO_2. All components of the system are maintained at 37°C in a thermostatically controlled enclosure.

2.5 Platelet functional studies

2.5.1 Platelet aggregation

Platelet aggregation studies were performed in a PAP4 aggregometer (Bio-Data Corporation) in which 0.5 ml aliquots of platelets were incubated (2 min) with stirring at 37°C before the direct addition of agonist. Changes in transmittance were compared to autologous PPP as the control. Prior to aggregation experiments, washed platelets were harvested by centrifugation (280g, 20 min) and resuspended in autologous PPP. Agonists used were ADP (10 μM), thrombin (10 units ml^{-1}), and U46619 (10 μM). In reconstitution experiments freshly washed platelets in HBSS w/o, had TPO (5 ng ml^{-1}; Peprotech), PDGF (10 ng ml^{-1}; Peprotech), IGF-1 (100 ng ml^{-1}; Peprotech), or BSA (4 mg ml^{-1}) directly added to final concentrations, followed by incubation at 37°C (2 h).

2.5.2 Platelet adhesion and cell spreading on collagen

Glass microscope slides were coated with either collagen I or IV solutions (100 mg ml^{-1}) in PBS. After 30 min the slides were washed with PBS before applying a volume of suspended platelets in cultured media. Following incubation (20 min), the slides were flicked free of media and non-adherent cells and snap-frozen with ice-cold methanol and acetone (1:1). The slides were then air-dried before staining with phalloidin-TRITC in PBS (2 μg ml^{-1}) and anti-CD41-FITC (1:1000, Serotec). Slides were examined by epifluorescent microscopy (Zeiss).

2.6 Immunolabelling and flow cytometric analysis

2.6.1 Analysis of platelet cell surface proteins

Immunofluorescent labelling of intact platelets for surface expression of CD41 (clone PM6/248; Serotec), CD42a (clone GRP-P; Serotec), CD61 (clone BL-E6; Caltag Laboratories), or CD62P (clone CRC81; Caltag Laboratories) was typically performed by resuspending 5 µl of cultured platelets with 40 µl of the appropriate fluorophore-conjugated Ab diluted 1:1000 with 10% NBCS in PBS (10 min), before adding 400 µl of FACS sheath fluid and sampling by flow cytometry using a FACScaliber and CellQuest software (Becton Dickinson). Phosphatidylserine (PS) exposure by intact platelets was determined by resuspending 5 µl of cultured platelets in 400 µl of HBSS containing 2 mM Ca^{2+} and FITC-conjugated annexin-V (0.3 µl ml^{-1}; Boeringer). All labelling steps were maintained at 4°C.

2.6.2 Analysis of platelet intracellular proteins

Intracellular immunolabelling of the Bcl-2 family of proteins was performed following fixation and permeabilization of the platelets with PermeaFix (Ortho Diagnostics), typically using 1 ml per 3 x 10^8 platelets on ice (30 min). Excess fixative was then removed with two washes of HBSS w/o (280g, 5 min) before resuspending with 10% NBCS in PBS at 3 x 10^8 ml^{-1}. 5 x 10^6 platelets were then incubated overnight with neat antibodies to Bak (1 µl), Bax (1 µl), Bcl-2 (1 µl), Bcl-x (1 µl), and Mcl-1 (1 µl) (all Pharmingen) or their appropriate negative controls. All primary Abs were detected with FITC-conjugated $F(ab')_2$ fragments of sheep anti-rabbit pAbs or goat anti-mouse pAbs.

2.6.3 Analysis of platelet mitochondrial transmembrane potential

Immunofluorescent labelling of intact platelets for inner-mitochondrial membrane potential ($\Delta\psi M$) was performed by adding 5 µl of platelets to 400µl of PBS w/o Ca^{2+} containing JC-1 (10 µg ml^{-1}; Molecular Probes), followed by incubation at 37°C (20 min), before sampling by flow cytometry.

Deliberate uncoupling of the respiratory chain with subsequent loss of platelet $\Delta\psi M$ was achieved by direct addition of the protonophore mCCCP (10 μg ml^{-1}).

2.6.4 Enumeration and functional evaluation of culture derived platelets

Culture supernatants containing platelets were separated by centrifugation (300 g, 2 min) and platelets enumerated by reference to a fixed count of 10 μm polystyrene beads (Polysciences). Functional platelets were distinguished from cellular debris by the characteristic increase in side scatter on shape change following thrombin (1 U ml^{-1}) or ADP (3 μM) treatment, and an absence of annexin-V binding (0.3 μl ml^{-1}; Boeringer) to the shape changed population. All conditions contained propidium iodide (10 μg ml^{-1}), with events staining positive excluded from subsequent analysis. Transient calcium flux was detected using FLUO-3-AM (10 μm; Molecular Probes). Culture derived platelets were separated as above, incubated at 37°C with FLUO-3-AM (20 min), and stimulated with agonists as above before sampling by flow cytometry.

2.7 SDS-PAGE and protein blotting

2.7.1 General considerations

For analysis of protein changes on induction of cell death, protein was analysed on the basis of equal numbers of extracted cells, rather than on the amount of protein loaded. Using a ratio of 100 platelets to 1 Jurkat cell resulted in lysates of comparable protein content as assayed by the BCA protein assay (Pierce). The assay is based on the ability of protein to quantitatively cause a reduction of Cu^{2+} to Cu^{+}, and bicinchoninic acid (BCA) to chelate Cu^{+} forming a purple compound, which can be measured by spectrophotometry (562 nm). Samples were diluted 1 in 10 in dH_2O and 10 μl incubated with 200 μl of test solution (30 min, 37°C) prior to analysis using an automated plate reader (Anthos). Samples were assayed in triplicate and standard curves formed using pre-made BSA standards. For comparison of relative protein levels between cells under constitutive conditions extracts were analysed on an equal protein loading basis. This distinction was made so that any

"global" loss of cellular protein witnessed on induction of cell death would not result in the apparent "false amplification" of other proteins when adjusting cell lysates for equal protein loading. Lysates were prepared by resuspending pelleted cells into ice-cold general cell lysis buffer with protease inhibitors (PMSF added fresh just before use) and incubated on ice (10 min). Cells were disrupted by passing through a 25 G needle ten times, followed by centrifugation (10,000g, 10 min, 4°C) retaining the supernatant. Lysates were added directly to four times laemlli buffer and boiled (97°C, 3 min) before long-term storage at -20°C. Molecular weight markers used were Rainbow Markers, SDS-7B, or BenchMark (Gibco Life Technologies).

2.7.2 Detection of cytochrome-C

For cytochrome-C detection, washed platelets were resuspended in ice-cold 10 mM Hepes buffer, pH 8.0 (containing 1mM EDTA, 1 mM 1,10-phenanthroline, 1mM phenylmethylsulphonylfluoride, 1 mM benzamidine, 10 μM pepstatin, 10 μM leupeptin, 10 μM antipain,), and lysed following two rounds of freeze-thaw. The lysed platelets were then centrifuged at 10,000g to yield a cytosolic supernatant (S10) and the pellet resuspended in lysis buffer before centrifuging again and dissolving the pellet in 1% TX-100 and re-centrifuging at 10,000g to yield soluble protein from intact mitochondria (T10). The S10 and T10 fractions were then pre-cleared with a control IgG and Protein-G agarose before immunoprecipitating cytochrome-C with clone 6H2.B4 (Pharmingen) and boiling in SDS sample buffer (Laemmli). Cycloheximide (20μg ml^{-1}; Calbiochem) treated Jurkats were used as a positive control and prepared identically. Cytochrome-C was detected using clone 7H8.2C12 (Pharmingen)

2.7.3 Cell free caspase-3 activation

For detection of caspase-3, Jurkats and fresh platelets were resuspended in ice-cold 20 mM Hepes buffer, pH 7.5 (containing 1.5 mM MgCl, 1mM EDTA, 1 mM EGTA, 1 mM DTT, 100 μm PMSF, 10 μg ml^{-1} leupeptin, 2 μg ml^{-1} aprotinin), and incubated on ice (10 min). In addition platelets were subjected to three cycles of freeze-thaw, before disruption of both cells by passing through a 25

G needle ten times. Cell free apoptosis was initiated by addition of cytochrome-C (10 μg ml^{-1}) and dATP (1 mM) to lysates, followed by incubation at 37 °C. In reconstitution experiments, human recombinant caspase-9 (0.1 U ml^{-1}; Chemicon) was directly added to lysates. Control sample lysates had buffer alone added. At indicated time points aliquots were removed and directly added to eppendorfs containing 4 times Laemmli buffer, and immediately boiled (3 min).

2.7.4 Two-dimensional electrophoresis

Two-dimensional electrophoresis (2D-E) was conducted using the Multiphor II electrophoresis unit, Immobiline DryStrip IPG gel kit, and additional ancillary reagents and equipment (all Amersham Pharmacia Biotech). 2D-E and the use of the Multiphor system is described in extensive detail and at great length elsewhere (Berkelman and Stenstedt 1998), and is unnecessary for this thesis. Briefly, 50 μg of cell derived protein prepared in 2D lysis solution was mixed with IPG rehydration buffer to a final volume of 200 μl. An 11 cm pH 3-11 IPG strip was placed into an Immobiline DryStrip reswelling tray, the sample and rehydration solution applied, everything overlayed with IPG cover fluid, and allowed to rehydrate overnight. The rehydrated IPG strip was transferred to a pre-cooled Multiphor flatbed electrophoresis unit, aligning the acidic end towards the anode, electrodes applied, and everything overlayed with IPG cover fluid. Isoelectric focusing is conducted at very high voltages (~3500 V), and very low currents (<1mA) due to the low ionic strength within the IPG strip. Voltage was stepped from 300 V (2 h), through 800 V (2 h), 1500 V (4 h), to a final voltage of 3500 V, which was maintained overnight (~16h). Focused IPG strips were equilibrated with an SDS buffer system to allow second dimension separation. Equilibrated strips were applied to the stacking gel of a typical vertical gel system, overlayed with 1% agarose, and electrophoresed and immunoblotted in a conventional manner.

2.7.5 Immunoblotting and detection

Electrophoresed proteins were transferred to PVDF membranes using a cooled "wet" transfer tank system. Typically transfers were conducted at 2 mA per cm^{-1}, whereby proteins less than 80 kDa fully transferred after 1.5 h. Larger proteins, such as APAF-1, usually required 2.5 h. Membranes were blocked with either 2% casein or 5% non-fat dried milk powder (Marvel) for 1 h, before incubation at 4°C in a rolling tube with primary Ab overnight. Primary antibodies were typically used at a dilution of 1:1000 in blocking buffer, but were titrated where necessary. Again were necessary due to high levels of non-specific antibody binding Tween-20 was added at a concentration of between 0.05-0.1%. Membranes were thoroughly washed with PBS or PBS/Tween, before incubation with an HRP-conjugated secondary antibody in blocking buffer for 1h. For development by the 4-chloro-1-napthol method, secondary antibodies were used at 1:1000, whilst for development by ECL plus a dilution of 1:5000 was more typical, but again titration of optimum levels was conducted when necessary.

For development by the 4-chloro-1-napthol method, 10mg of 4-chloro-1-napthol was dissolved in a minimal volume of ethanol and diluted to 50ml with development buffer. Washed stained membranes were incubated in this solution with agitation and the development reaction initiated with the addition of 50µl of H_2O_2 (30% v/v). Development was stopped by washing the membrane free of developer solution with PBS. ECL plus (Amersham Pharmacia Biotech) was conducted as per the manufacturers instructions. ECL signals were detected using either a phosphoimager (Molecular Dynamics) equipped with image analysis software (Molecular Dynamics), or following exposure to autoradiographic film (Amersham Pharmacia Biotech). Primary Abs used were caspase-3 pAb, caspase-9 pAb, caspase-9 mAb (clone B40), APAF-1 pAb (all Pharmingen), Bid pAb (BioSource International), and β-actin mAb (clone AC-15).

2.8 Analysis of platelet plasma membrane integrity

2.8.1 Flow cytometric analysis using phalloidin-FITC as a vital dye

Due to platelets anucleate nature more typical DNA binding vital dyes were unsuitable. Phalloidin is a natural filamentous-actin binding toxin extracted from the mushroom amanita phalloides, is extremely hazardous, and should be used with great caution. Fresh or washed aged platelets, 5µl, were added to 500µl of PBS containing phalloidin-FITC (2 µg ml^{-1}) and incubated at RT (10 min). Platelets were washed once by centrifugation (280g, 5 min), and resuspended in PBS before analysis by flow cytometry. As a positive control for saturated actin phalloidin binding, platelets were permeabilised on ice (30 min) using PermeaFix (Ortho Diagnostics) before staining.

2.8.2 Spectrophotometric assay for Lactate Dehydrogenase

Fresh washed platelets were incubated overnight and pelleted by centrifugation (280g, 20 min). The supernatant was removed and the pellet resuspended to the same volume in HBSS and sonicated. Stock solutions of NADH (0.2 mM) and sodium pyruvate (1.6mM) were freshly prepared in a Tris (81.3 mM)/NaCl (203.3mM) buffer, pH 7.2. The assay was initiated by the addition of platelet supernatant or sonicate (40 µl) to a quartz silica cuvette maintained at 37°C and containing 420 µl of NADH and 80 µl pyruvate. LDH activity was measured as the time-dependent and pyruvate-dependent decrease in the absorbance of NADH at 339nm using a spectrophotometer (Cecil). LDH activity was expressed as µmoles of NADH consumed per min per ml of platelet supernatant or sonicate.

2.9 Extraction of genomic DNA for analysis of fragmentation

During the extraction of genomic DNA the mixture must never be vortexed, vigorously shaken, or pipetted thorough fine tips due to the shearing that will occur, with the result that the genomic DNA will run on the gel as a continuous smear. Approximately 2 x 10^6 cells were collected

by centrifugation (220g, 5 min) and resuspended in 1 ml of nuclear lysis buffer. Proteinase K was added (30 µl of 10 mg ml^{-1} stock) and the solution gently mixed, followed by the addition of 20 µl of SDS (10%), gentle mixing, and incubation at 55°C for at least 3 h, or alternatively overnight. This solution was gentle mixed with 1 volume of 1:1 phenol:chloroform and centrifuged (10,000g, 1 min), to form two layers. The top layer was gently removed ensuring the interface, consisting of denatured protein, was not disturbed. DNA was precipitated by addition of 1 volume of ice-cold isopropanol and several inversions of the tube. DNA was pelleted by centrifugation (10,000g, 15 min), the supernatant aspirated, the pellet gently washed with 70% ethanol, and the tube allowed to air dry. Genomic DNA was redissolved in 50 µl of TE at 37°C for several hours. DNA concentration and purity was determined using a spectrophotometer (Cecil) at 260 nm and 260/280 nm respectively. Typically, 1 µg of genomic DNA was loaded onto a 1.2% agarose minigel and electrophoresed at 140 mA for 45 min. DNA was visualised under UV light with ethidium bromide staining.

2.10 Microscopy

2.10.1 Phase and epi-fluorescence microscopy

Images were captured on an inverted microscope (Zeiss) equipped with an air cooled CCD camera (CoolSnap) and OpenLab 3.0 image acquisition software (ImproVision). Final overlayed image were produced using PhotoShop 6.0 (Adobe). Live cell suspensions were incubated *in situ* in Willco micro dishes (Intracel) and directly imaged through the 0.17 cover glass bottoms. Active caspases were detected using CaspaTag as instructed (Intergen). Briefly, cultured control or treated MKs were gently and slowly transferred to an eppendorf using a wide bore pipette and incubated at 37°C (30 min) with CaspaTag diluted to a final 1x solution. Following this incubation period all MKs were found to have pelleted, thus enabling supernatant to be aspirated and the MKs to be gently resuspended into CaspaTag wash buffer. MKs were again allowed to pellet, supernatant removed and cells resuspended before analysis. Although slow this method allowed a greater recovery of intact proplatelet bearing MKs.

Mitochondrial membrane potential was qualitatively determined using JC-1 (Molecular Probes) at a final concentration of 5 μg ml^{-1}, following incubation at 37°C (20 min). Nuclear morphology was detected by the direct addition of Hoechst 33342 (2μg ml^{-1}) to culture medium. Confocal images were captured on a Leica TCSNT microscope and software system. Assessment of proplatelet $\Delta\psi M$ by confocal microscopy was with a limited number of Z-axis scans due to cell and proplatelet motility. MKs were stained for cell surface Fas using a murine anti-Fas IgM mAb (clone CH.11). Cells were fixed with formaldehyde (2%) on ice (30 min), before washing away fixative by centrifugation (180g, 5 min), followed by staining with CH.11 (50 ng ml^{-1}) on ice (1 h). Primary antibody was washed away and detected using an AlexaFluor (Molecular Probes) anti-murine secondary antibody (1:1000), following incubation on ice (1 h).

2.10.2 Transmission electron microscopy

Cells or culture supernatants were directly fixed with 2.5% glutaraldehyde (EM grade I) in 0.1 M sodium cacodylate buffer for at least 1 h at RT. Fixative was removed with one wash of 0.1 M sodium cacodylate buffer, cells pelleted, and overlayed with a minimal volume of fresh PPP for at least 1 h at RT. A fibrin plug was formed around the pellet by overlaying the PPP with a minimal volume of fixative, resulting in pseudo coagulation. Plugs were subsequently treated as normal resected tissue and processed with osmium tetroxide, lead citrate, araldite embedding and ultra thin sectioning (60nm). Samples were analysed on a Philips CM150 TEM.

Chapter 3 - Platelet Cell Death

Introduction

Close to 2 x 10^{12} platelets circulate in the average adult human, and around a tenth of these are efficiently and safely cleared from the circulation every day. Representing an anucleate cell with a defined *in vivo* lifespan (Stuart *et al* 1975), platelets are of critical importance for the maintenance of normal haemostasis, but disorders of platelet number and function are common giving rise to a range of bleeding or thrombotic disorders, including stroke and myocardial infarction. Given this central role in normal mammalian physiology and disease, it is remarkable that there has been very little study of any potential constitutive death program that could account for platelet deletion *in vivo*. Many older studies have proposed platelets to passively lose surface markers of self through attrition within the circulation, leading to clearance mediated by the spleen (Greenberg *et al* 1975; 1979). Interestingly, platelets activated and reinfused into the circulation show an identical lifespan to control platelets, suggesting activation related platelet changes are dissociated from biochemical alterations leading to their clearance (Berger *et al* 1998; Michelson *et al* 1996). An important preliminary study (Vanags *et al* 1997) suggested that platelets may undergo an apoptotic program since increases in the expression of proapoptotic members of the Bcl-2 family of death regulating proteins were observed following treatment with ionomycin, a calcium ionophore which induces apoptosis in a range of cell types, most notably lymphocytic cells (Wyllie *et al* 1984; Squier and Cohen 1997). Interestingly, this study showed that ionomycin induced an increase in Bax and Bak, as well as the cell surface expression of PS, both representative of key features of apoptotic cell death.

Apoptosis represents a designation of a particular form of cell death defined by classical morphological and biochemical changes, and has attracted intense scrutiny as a self-contained and physiological program for deletion of unwanted cells (Kerr *et al* 1972; Wyllie *et al* 1980), eventually resulting in plasma membrane changes which lead to the key physiological outcome of apoptosis – the non-phlogistic recognition and uptake of the intact dying cells by phagocytes (Martin *et al* 1996). However, the study of apoptosis has been dominated by experiments which have frequently relied on artificially induced death in transformed cells and which have rarely paid attention to their recognition and clearance by phagocytes. Nevertheless, these criticisms have been addressed in studies of primary

blood cells freshly isolated from healthy human donors (Ren and Savill 1998). For example, neutrophil granulocytes purified by methods designed to minimize artefactual activation (Haslett *et al* 1985) undergo constitutive apoptosis that clearly directs specific recognition by phagocytes (Savill *et al* 1989; 1993). Nevertheless, many other forms of cell death that do not meet the typical biochemical and morphological criteria of apoptosis have now been reported (Lavoie *et al* 1998; Mathiasen *et al* 1999; Foghsgaard *et al* 2001). These newly described programs of cell death highlight the various adaptations to the basic program nature has produced for utilisation in different cell types, tissue locations, death stimuli, and of course underscores the strong redundancy in a cells ability to die safely.

To investigate whether platelets could undergo a constitutive apoptotic-like death program, freshly isolated platelets were cultured for up to 24 h at 37°C. In the presence of autologous plasma we observed an increase in the levels of proapoptotic Bax and Bak, in keeping with an earlier report (Vanags *et al* 1997), and consistent with the idea that platelets might engage a constitutive death program on aging *ex vivo*. In support of this, we also observed a constitutive loss of key platelet functional responses, including agonist-induced aggregation and spreading on collagen. By washing platelets free of their native plasma, a putative source of survival factors, not only was loss of function accelerated but there was also revelation of a cell death program characterized by cytoplasmic condensation as revealed by TEM, display at the cell surface of phosphatidylserine and P-selectin, but with retention of plasma membrane integrity, and specific recognition and clearance by phagocyte scavenger receptors.

3.1 Cultured platelets exhibit increased levels of pro-apoptotic Bcl-2 family members

An important determinant of whether a cell will undergo apoptosis is its intracellular balance between anti-apoptotic members of the Bcl-2 protein family such as Bcl-2 and Mcl-1, and proapoptotic members such as Bax and Bak (White 1996; Reed 1998). The possibility that platelets might be able to undergo an apoptosis-like death was first raised by Vanags *et al* (1997) who reported that ionomycin, a calcium ionophore which triggers apoptosis in many cell types (Wyllie *et al* 1984; Squier and Cohen 1997; Gwag *et al* 1999), caused an increase in the expression of proapoptotic Bax and Bak, but not Bcl-2. In order to extend the findings of Vanags *et al* we decided to study a different model system in which apoptosis-like death might occur. Granulocytes "aged" in culture undergo constitutive death that is accelerated in the absence of survival factors (Mangan *et al* 1991; Lee *et al* 1993; Dibbert *et al* 1998) and is correlated both with increased levels of Bax (Manfredini *et al* 1998; Weinmann *et al* 1999), but not Bak (Bazzoni *et al* 1999), and decreased levels of Mcl-1 (Moulding *et al* 1998). We therefore tested the hypothesis that, as with granulocytes, prolonged culture of platelets might also lead to an increase in the expression of pro-apoptotic Bcl-2 family members and a susceptibility to apoptosis-like death.

3.1.1 Platelets aged in plasma show increased expression of Bax and Bak

Following intracellular staining and assessment by flow cytometry we confirmed that freshly isolated platelets expressed Bax, Bak, and Mcl-1, but intriguingly not Bcl-2 (Figure 3.1). The ability of each antibody to recognise its antigen by flow cytometry was verified with appropriate control cell lines (Magowan 2001). Interestingly, the levels of immunodetectable Bak and Bax increased by 3.4 and 2.4-fold respectively as platelets were aged for 18 h in citrated plasma, while no significant changes in Mcl-1 were apparent (Figure 3.2). In keeping with studies of ionomycin stimulation of platelets (Vanags *et al* 1997), these data suggest that platelets cultured for 18 h can adopt a more proapoptotic balance and further suggest that aging in culture, as originally reported for neutrophils (Savill *et al* 1989), might be a useful model for studying platelet cell death.

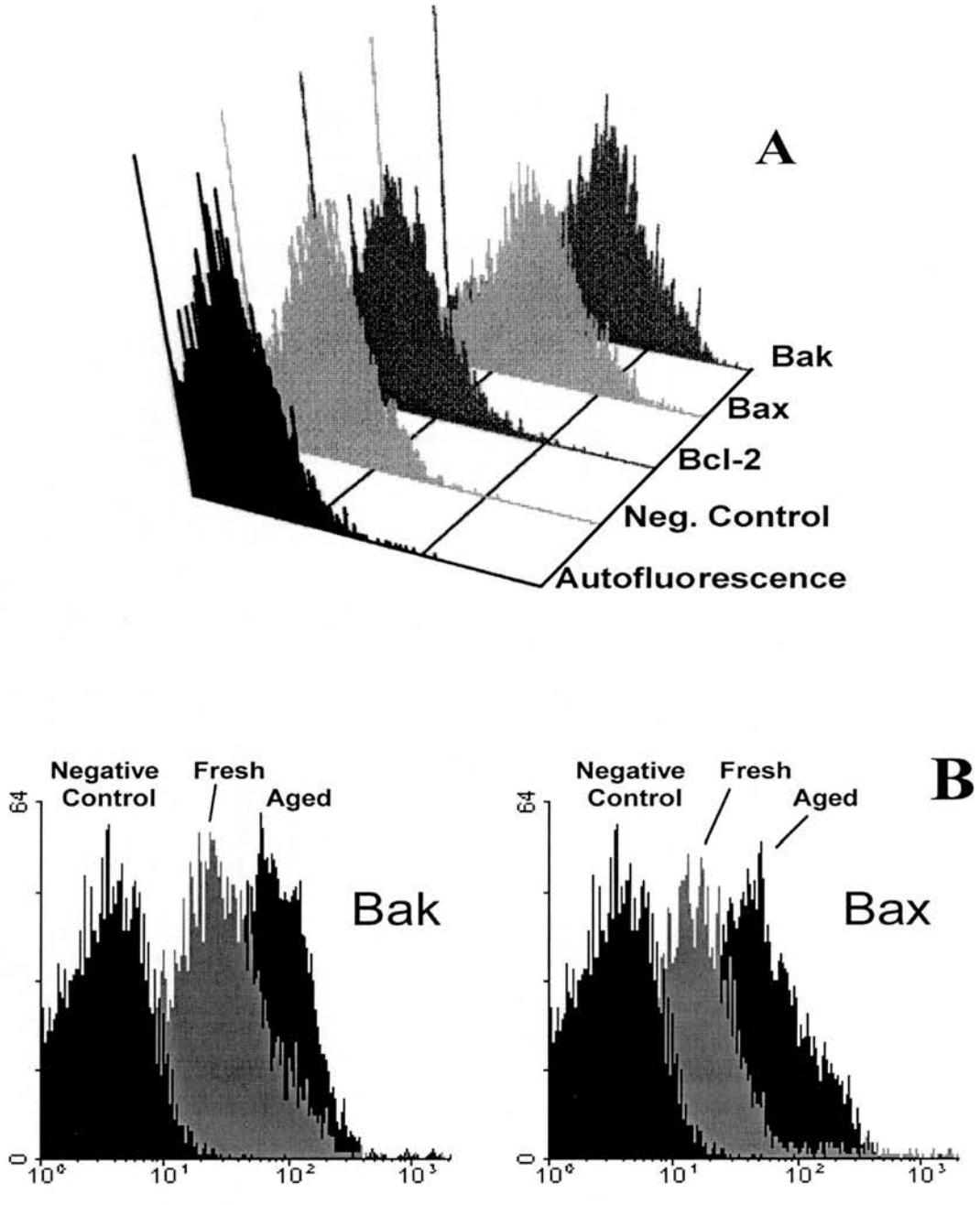


Figure 3.1: The proapoptotic balance of Bcl homologues is accentuated in aged platelets. (A) Typical immunofluorecence flow cytometry histograms of PermeaFixed cells which demonstrate that freshly isolated platelets express the proapoptotic Bcl-2 homologues Bak and Bax, but not the anti-apoptotic Bcl-2. The negative controls for mouse (Bcl-2) and rabbit (Bak, Bax) Abs were superimposable and no different from autofluorescent controls, confirming an absence of non-specific binding. **(B)** Overlays of representative histograms showing the shift in fluorescence for Bak and Bax expression between freshly isolated and aged platelets. Autofluorescence and control irrelevant staining for fresh and aged platelets were essentially indistinguishable.

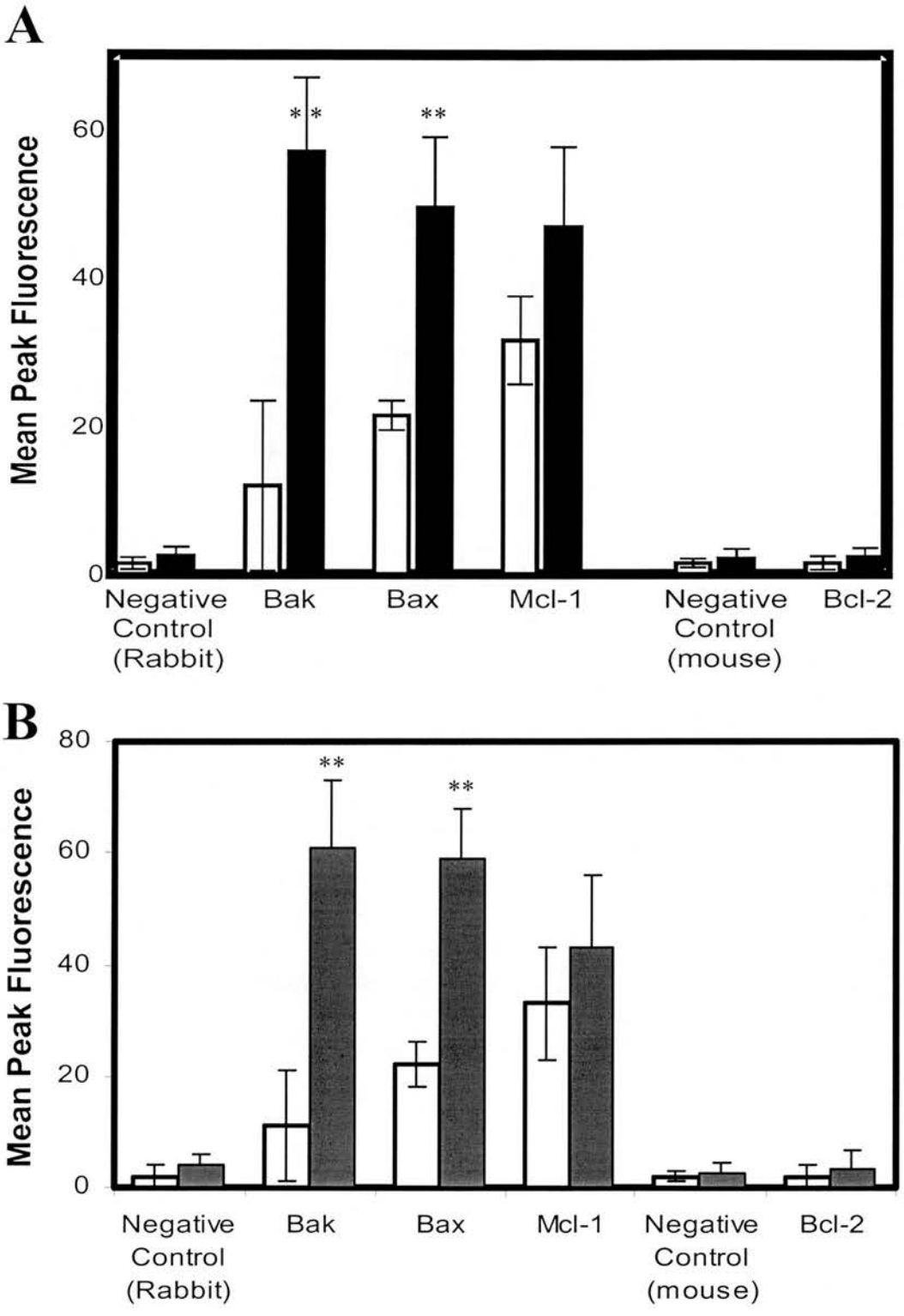


Figure 3.2: The proapoptotic balance of Bcl homologues is accentuated in aged platelets. The mean peak fluorescence for various members of the Bcl-2 family are presented as the arithmetic mean ± 95% confidence interval for n = 4 separate experiments (4 different donors). Each experiment was performed in duplicate on PermeaFixed samples with fresh citrated platelets (open columns), platelets cultured in the presence of citrated plasma for 18h **(A)** (solid columns), or platelets washed and cultured without plasma for 6 h **(B)** (solid columns). ** denotes $p < 0.02$ as determined by ANOVA.

3.1.2 Washing platelets free of plasma factors accelerates the increase in Bax or Bak

Since plasma represents a potential source of exogenous survival factors we went on to seek evidence that plasma deprivation might accelerate and therefore reveal other biochemical changes indicative of a constitutive death program, previously overlooked in short-term culture experiments on platelets. Using a washing protocol based on diluting the plasma 1:5 with HBSS w/o Ca^{2+}, in the presence of 4mM EDTA, the majority of platelets (74 ± 3 %) could be pelleted and subsequently resuspended in HBSS w/o followed by incubation at 37 °C. Platelets aged under these conditions exhibited increased levels of Bax and Bak comparable to those observed for platelets aged in the presence of plasma, however the change occurred much more rapidly, seen within 6 h of culture (Figure 3.2).

3.2 Aged platelets exhibit impaired function

Platelets possess many of the functional responses exhibited by other inflammatory blood cells and a key feature of apoptotic leukocytes cultured overnight in the presence of serum is loss of the ability to respond to external stimuli and subsequently mount pro-inflammatory responses (Whyte *et al* 1993). A range of easily detectable platelet functional responses exist including aggregation, shape-change, filopodia extension on collagen, intracellular calcium flux and serotonin reuptake, a subset of which we used to assess whether platelets lost functionality on aging.

3.2.1 Fresh platelets undergo normal aggregatory responses when stimulated by agonists

With the use of an aggregometer, which measures the light transmittance of a stirred platelet suspension, we confirmed that treatment of freshly isolated (viable) platelets with 10 μM ADP resulted in an immediate shape change, observed as a slight decrease in light transmittance (upward deflection in Figure 3.3; arrow), followed by an irreversible decrease in light transmittance that was indicative of a full aggregation response. By reducing the concentration of ADP to 3 μM, and in accordance with the biphasic nature of platelet aggregation (MacMillan 1966), we observed a reversal

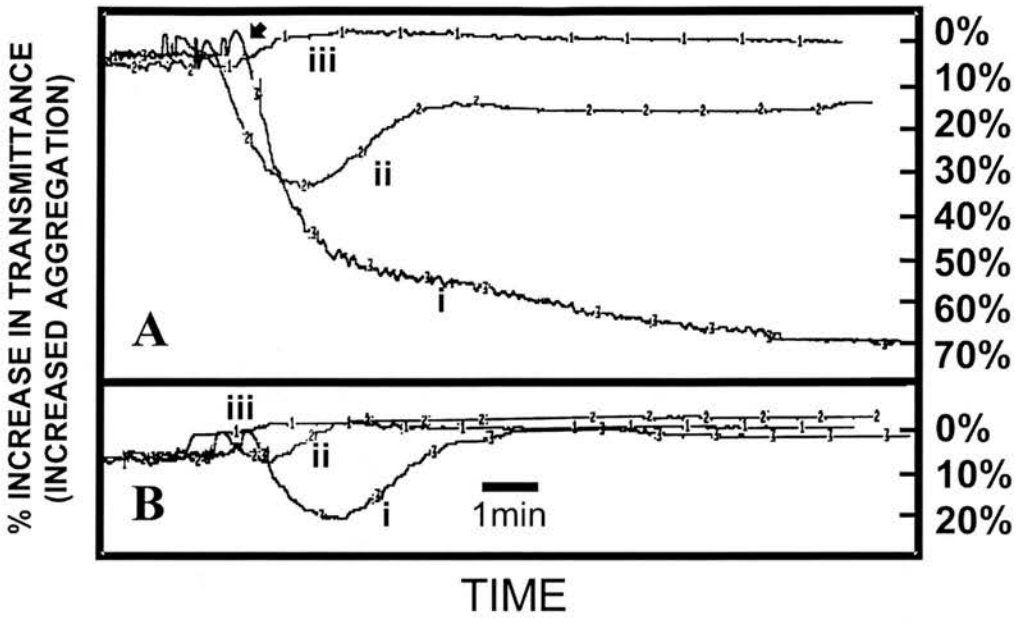


Figure 3.3: Agonist-induced aggregation is down regulated in aged platelets. Freshly isolated platelets **(A)** or platelets aged in citrated plasma for 24 h **(B)**, were monitored for changes in light transmittance over a 10 min period following activation with ADP to a final concentration of 10 μM **(i)**, 3 μM **(ii)**, and 1 μM **(iii)**. The upward deflection in the traces (arrow) following the addition of ADP is indicative of shape change, while increases in transmittance are indicative of reversible **(ii)** or full aggregation **(i)**. The chart recorder moved with a 1 min interval represented by the horizontal bar in **(B)**.

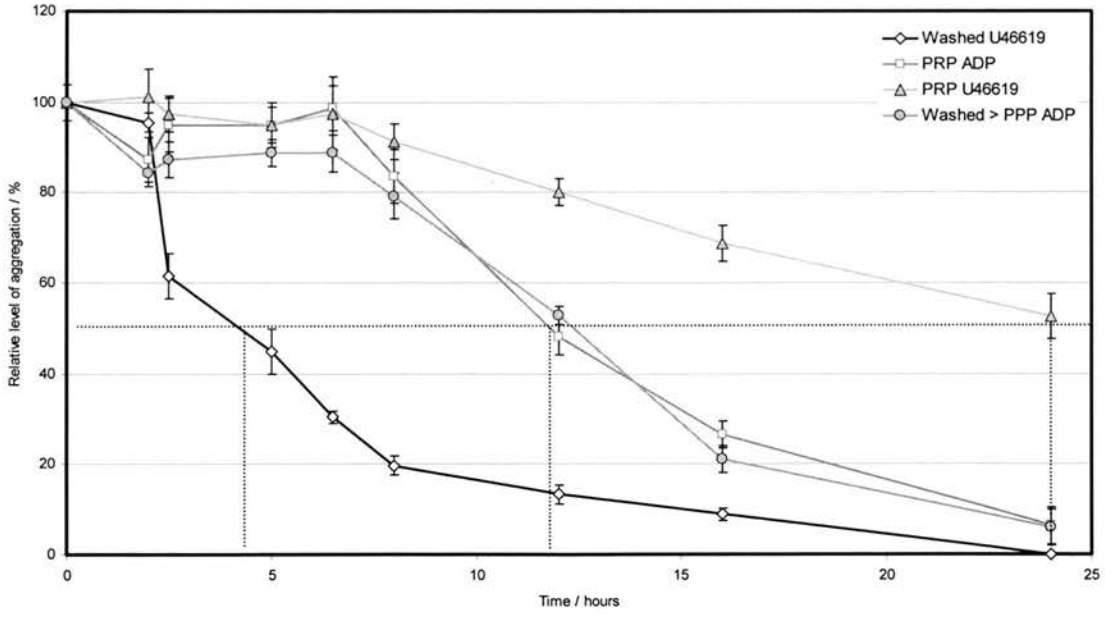


Figure 3.4: Time course for loss of agonist induced aggregation. PRP, washed platelets, or washed platelets resuspended into PPP were incubated at 37°C and assayed for the level of aggregation at the times indicated. Platelets were stimulated with U46619 (10 μM) or ADP (10 μM) as indicated. Data is reported as % aggregation relative to control levels (typically 70% maximum). Data represents mean ± one S.D. of n = 3.

of the initial wave of aggregation due to the absence of endogenous agonists such as ADP being secreted by the weakly activated platelets (Figure 3.3). In the presence of 1 μM ADP there was no evidence of aggregation although shape change, which is arguably the most sensitive response of platelets, persisted (Figure 3.3).

3.2.2 Platelets aged in the presence or absence of serum lose aggregatory responses

In contrast, we were able to confirm that platelets aged over a 24 h period in plasma lost the ability to aggregate ($t_{1/2}$=12 ± 2h), but not to undergo a shape change in response to ADP (Figure 3.3). The loss of response by aged platelets was also observed with the endoperoxide analogue and powerful platelet agonist U46619 (Main and Pearce 1978), although aggregation was still detected following 24 h of culture ($t_{1/2}$=24 ± 3h)(Figure 3.4). In keeping with the hypothesis that plasma might contain exogenous survival factors that normally retard constitutive platelet death, we found that the loss in aggregatory response to ADP was markedly accelerated when platelets were washed and cultured in the absence of plasma ($t_{1/2}$ = 55 mins ± 15 mins)(Figure 3.5). Similarly, the loss of response to U46619 was accelerated under these conditions ($t_{1/2}$ = 4 ± 0.7 h)(Figure 3.4). This rapid loss in ADP-induced aggregation by washed platelets was prevented and returned to rates comparable to those of unwashed platelets maintained in plasma by reconstituting the washed platelets with platelet-poor plasma (PPP) ($t_{1/2}$ =12 ± 2h) (Figure 3.4). This indicated that the loss in platelet response was not dependent on the washing procedure per se but rather on the absence of factors within plasma that normally suppress the observed loss of responsiveness.

3.2.3 Known survival factors for other cell systems fail to reverse loss of function

To extend these findings we assessed whether known survival factors from other cell systems, such as PDGF (Harrington *et al* 1994), and IGF-1 (Rodriguez-Tarduchy *et al* 1992), or the megakaryocyte differentiation factor thrombopoietin (Lok *et al* 1994; deSauvage *et al* 1994), could attenuate the loss of the ADP aggregatory response that occurred after 2 h with washed platelets. Freshly washed platelets were cultured in the presence or absence of these factors at concentrations

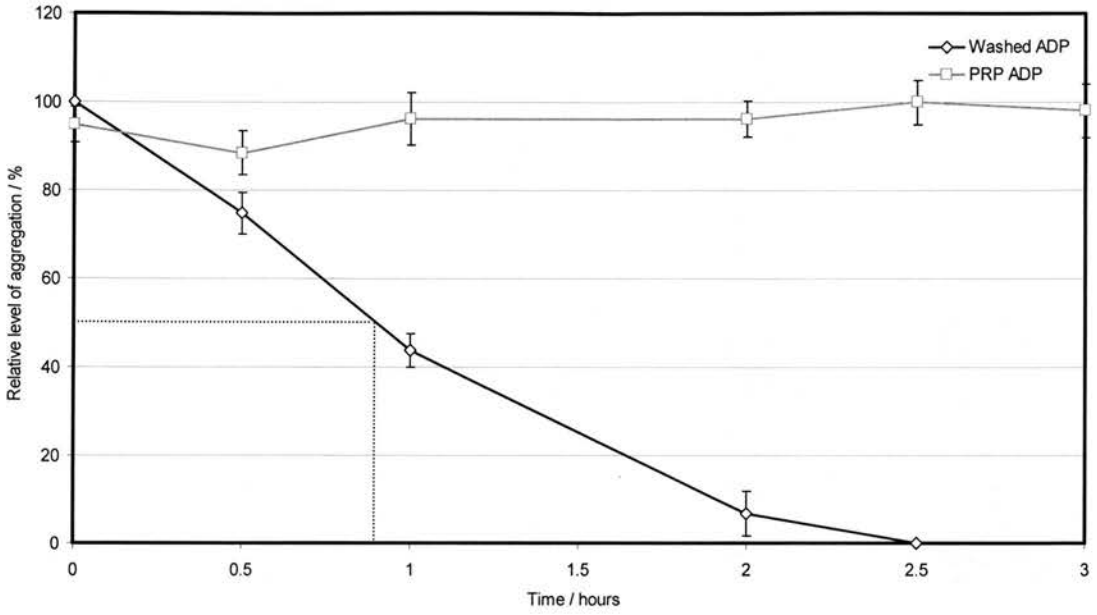


Figure 3.5: Time course for loss of agonist induced aggregation. PRP or washed platelets were incubated at 37°C and assayed for the level of aggregation at the times indicated. Platelets were stimulated with ADP (10 μM). Data is reported as % aggregation relative to control levels (typically 70% maximum). Data represents mean ± one S.D. of n = 3.

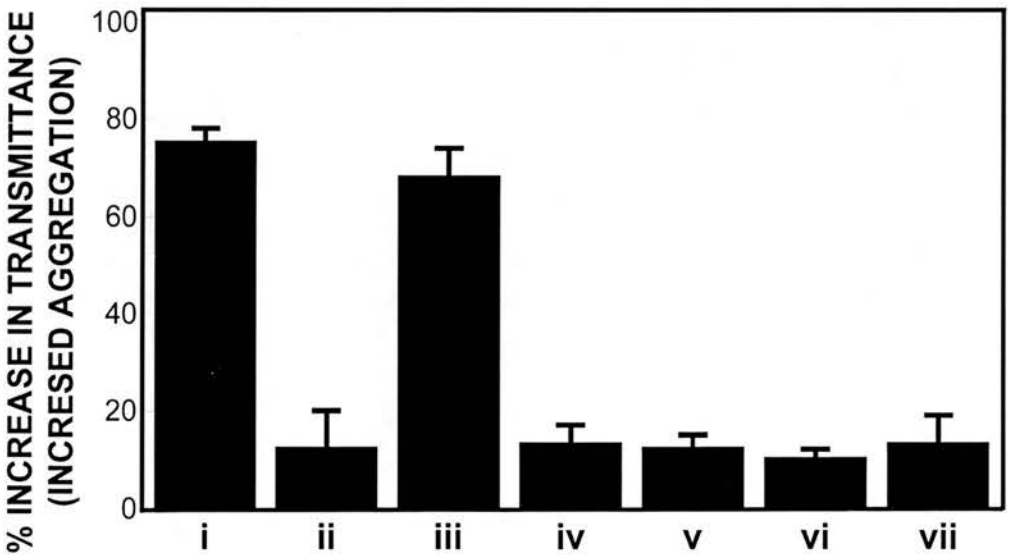


Figure 3.6: A panel of growth and survival cytokines fail to prevent the loss of aggregatory response. Freshly isolated platelets were maintained for 2 h at 37°C in either citrated plasma **(i)** or, after being washed free of plasma, in HBSS **(ii)** containing either thrombopoietin (5 ng/ml)**(iv)**, PDGF (10 ng/ml)**(v)**, IGF-1 (100 ng/ml)**(vi)** or BSA (4 mg/ml)**(vii)**. Alternatively, washed platelets were returned to PPP **(iii)**. ADP was added to 10 μM and change in light transmittance recorded for 10 min. Error bars represent the 95% confidence interval for n = 5 separate experiments.

typically seen to modulate death in other systems, and platelets assessed for their aggregatory capabilities after 2 h of culture. Figure 3.6 clearly shows no affect on the loss of the ADP response with these factors, and as stated previously returning washed platelets to their native PPP abrogated loss of function. As a control for oncotic effects we tested BSA at 4 mg ml^{-1} and found no protective effect. Initial investigations to characterize the putative soluble plasma survival factor(s) by dialysis have revealed the activity to be of 50 kDa or greater in molecular size and stable to long term storage at 4°C and –20°C.

3.2.4 Aged platelets fail to spread on collagen coated glass slides

Constitutive loss of platelet function on incubation at 37°C was further confirmed by assessing the ability of aged platelets to adhere and spread on collagen coated surfaces. In the context of haemostasis of the vasculature, platelets encountering collagen represents a powerful activation stimulus, usually indicative of exposure of the underlying basement membranes through damage to endothelial cells (Brass *et al* 1974). Direct contact of platelets to collagen surfaces results in the formation of lammelipodia and filopodia mediating a firm adhesion and providing a surface on which to recruit further platelets. Using the filamentous actin binding toxin phalloidin-FITC, microscopic examination of stained preparations revealed that freshly isolated platelets readily adhered and spread on both collagen I & IV coated glass slides, whereas aged platelets, whether cultured in the presence or absence of plasma protein, did not (Figure 3.7).

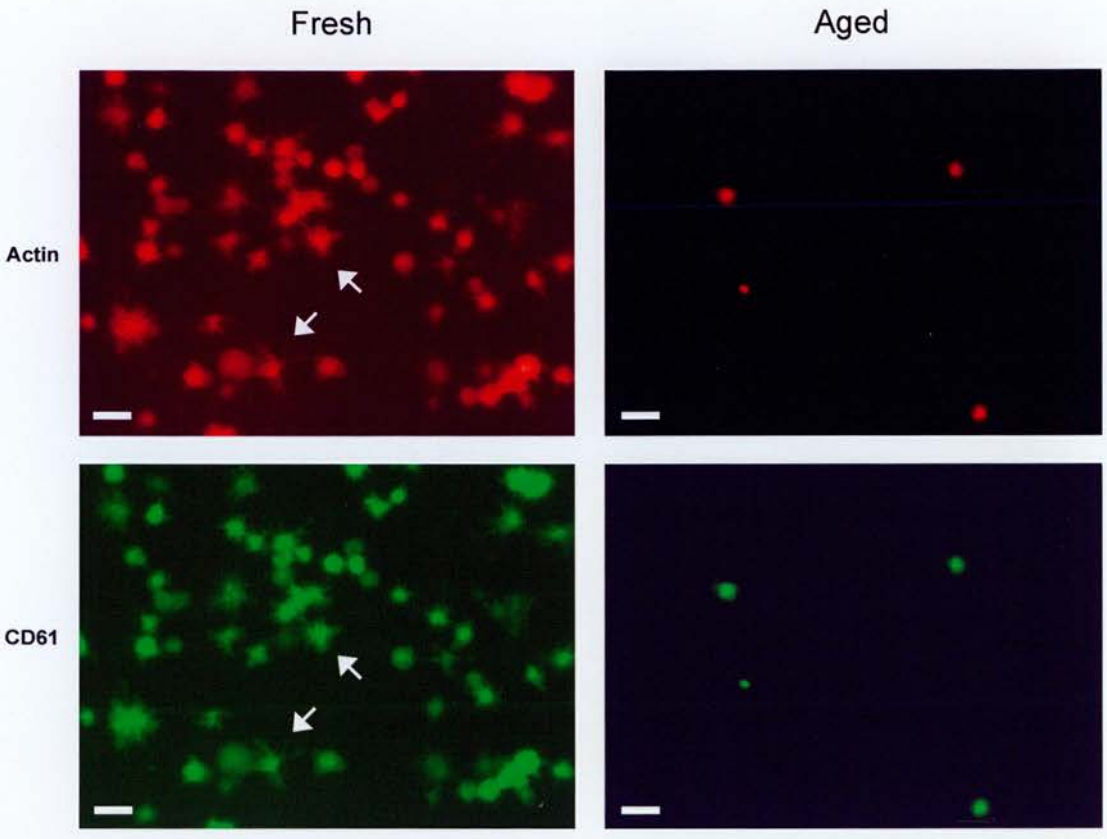


Figure 3.7: Aged platelets fail to adhere and spread on collagen. Fresh or aged platelets were incubated on collagen coated glass slides at 37°C to assess their ability to spread. Slides were fixed and stained with actin binding phalloidan-TRITC (orange), and the platelet specific CD61-FITC (green). The majority of fresh platelets can be seen to adhere and spread (arrow), whilst aged platelets rarely bind or spread. Scale bar represents 5 μm.

3.3 Aged platelets maintain plasma membrane integrity

Although down-regulation of cell function is a feature of cell death in other blood cells (Whyte *et al* 1993; Dransfield *et al* 1994; Dransfield *et al* 1995) it might also have reflected necrosis. Assessing necrosis is not straightforward in platelets as their small size precluded the use of vital dyes such as Trypan Blue, since admission of dye could not be confidently assessed by light microscopy. Similarly, the absence of a nucleus precluded the use of DNA staining vital dyes such as Hoechst 33342 and propidium iodide. Nevertheless, flow cytometry revealed that the forward and side scatter properties of fresh and aged platelets, whether cultured in HBSS or native plasma, were superimposable. This contrasted with the deliberate impairment of plasma membrane integrity by hypotonic lysis, thermal treatment, or mild acid treatment, which invariably resulted in appearance of debris and loss of platelets as assessed by forward and side scatter.

3.3.1 The filamentous actin binding phalloidin-FITC can be used as a vital dye

Evidence against necrosis being a confounding factor in the constitutive loss of platelet function was the use of phalloidin-FITC as an actin binding "vital dye". Using this reagent greater than 99% of both fresh and aged platelets excluded the dye when assessed by flow cytometry (Figure 3.8). As a positive control to reveal the potential saturation level of intracellular staining, aged platelets were permeabilised with the formaldehyde/saponin based fixative PermeaFix prior to staining with phalloidin-FITC. Following permeabilisation greater than 90% of aged platelets displayed a clear log order shift in fluorescence levels. Use of Epi-fluorescence microscopy confirmed that phalloidin-FITC appeared to stain the intracellular space of permeabilised platelets, supporting the use of this reagent as a novel vital dye. These data were a strong indication that aged platelets were capable of maintaining plasma integrity under the aging conditions employed in this study.

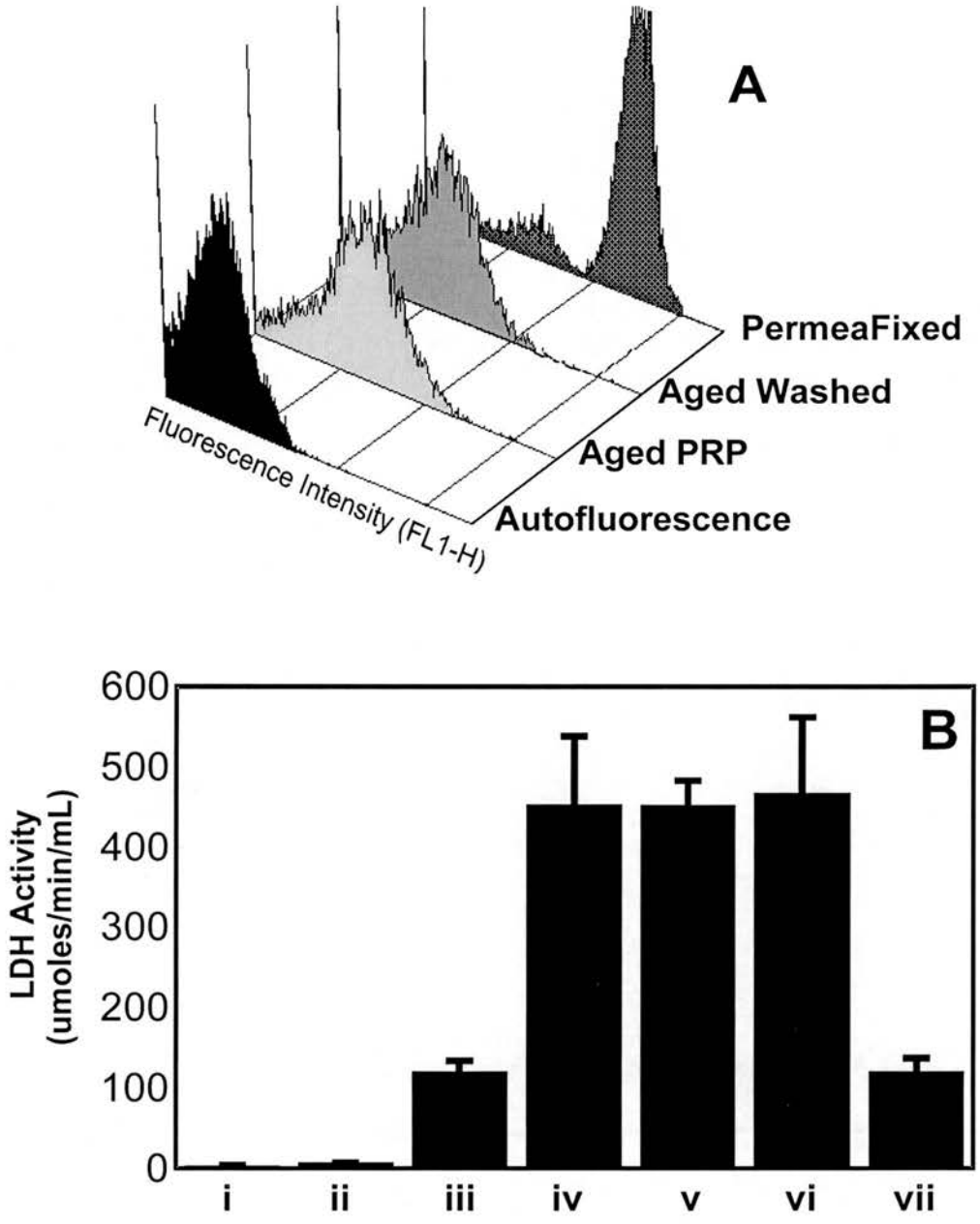


Figure 3.8 Aged platelets maintain plasma membrane integrity. (A) Flow cytometric analysis of platelet membrane integrity using actin-binding phalloidin-FITC as a vital dye. Platelets aged in the presence (aged PRP) or absence of plasma (aged washed) were incubated with phalloidin-FITC before flow analysis. The FL1 channel was set to the autofluorescence of unlabelled freshly isolated washed platelets. As a positive control for phalloidin-FITC staining, aged platelets cultured in the absence of serum were permeabilised with Permeafix. **(B)** Freshly isolated platelets were resuspended in HBSS and cultured in the absence of plasma for either 1 h or 18 h before separating cells from conditioned supernatants and resuspending the cells in an equivalent volume of fresh HBSS and sonicating. Clarified supernatants of fresh (i) and aged (ii) washed platelets, or lysates of fresh (iv) or aged (iii) were monitored for LDH activity. Lysates from fresh platelets were also incubated for 18 h in HBSS either on their own (v) or in the presence of erythrocytes (vi). The level of LDH in PPP is shown for comparison (vii). Error bars represent the 95% confidence interval of n = 4 separate experiments each done in duplicate.

3.3.2 LDH activity within culture supernatants confirms maintenance of membrane integrity

As extended confirmation of platelet integrity we assessed the level of lactate dehydrogenase (LDH) activity, an abundant cytoplasmic enzyme of platelets, within supernatants of cultured platelets (Figure 3.8). Reassuringly, less than 4% of total LDH activity was found in the supernatant of platelets aged for 18 h in HBSS in the absence of serum. However, the possibility that these low levels within supernatants were the result of enzymatic instability during prolonged culture, or cell surface endopeptidase mediated degradation of LDH was investigated. Therefore, soluble LDH within fresh platelet lysates was maintained under comparable conditions either on its own or with erythrocytes. These experiments revealed that soluble LDH was stable in culture over a 24 h period in both instances (Figure 3.8). The 75% loss in LDH activity in the cytoplasm of intact aged platelets appeared likely to reflect intracellular catabolism of retained LDH, or its inactivation by, for example, transglutaminases as opposed to leakage. Unfortunately, and given the high levels of LDH in plasma, we were unable to reliably assess the release of LDH from platelets aged in plasma. Taken together with the phalloidin data, these results provide strong evidence against the possibility that aged platelets may have undergone necrosis, but rather may have undergone a form of programmed cell death.

3.4 Transmission electron microscopy of aged platelets is suggestive of a cell death program

To further eliminate the possibility of necrosis or an activation phenomenon, we prepared samples for ultrastructural analysis by transmission electron microscopy (TEM). For cells in suspension there are two main preparation methods, pre- and post- fixation cell pelleting. The former will bind pelleted cells together by fixation of the plasma membranes within the pellet, whilst the latter requires formation of a fibrin clot around the pellet after fixation. Given the potential for artefactual platelet activation on manipulation we deemed it better to fix platelets in suspension, before pelleting and encapsulation within a fibrin plug. In addition this method essentially provides a

protein rich support-matrix allowing the pellet to be treated as resected tissue, producing excellent results.

3.4.1 Washing does not induce obvious evidence of platelet activation

Washed or untreated freshly isolated platelets exhibited characteristic discoid-like features of non-activated platelets (Figure 3.9). This was confirmed and easily contrasted to the presence of elongated filopodia, enlarged canalicular system and coalescence of granules, surrounded by a circumferential band of a constricting microtubular network, seen within deliberately activated platelets. A cross-section through both platelets revealed the typical distribution of dense bodies, α-granules, mitochondria, and glycogen particles, as indicated.

3.4.2 Aged platelets exhibit cytoplasmic condensation

Platelets aged in culture under conditions leading to loss of function exhibited dramatic morphological changes. Control platelets maintained in citrated plasma for 24 h exhibited few changes from the freshly isolated state, except that the majority of cells assumed a more spherical rather than a discoid shape (Figure 3.10). However, when aged for 12 h in HBSS in the absence of serum, when loss of function was complete, there was remarkable condensation of cytoplasm and granules with sub-membrane vacuolisation, with these features more pronounced at 24 h of culture (Figure 3.10). Interestingly, cultured platelets did not exhibit plasma membrane blebbing, a common feature of many cell types undergoing apoptosis, but not of granulocytes (Savill *et al* 1989). Furthermore, and in keeping with granule fusion with the plasma membrane as aged neutrophils progress to an intact late apoptotic state prior to secondary necrosis (Gilligan *et al* 1996), platelets aged for 24 h contained evidence for fusion of granules with the plasma membrane (Figure 3.10; arrows).

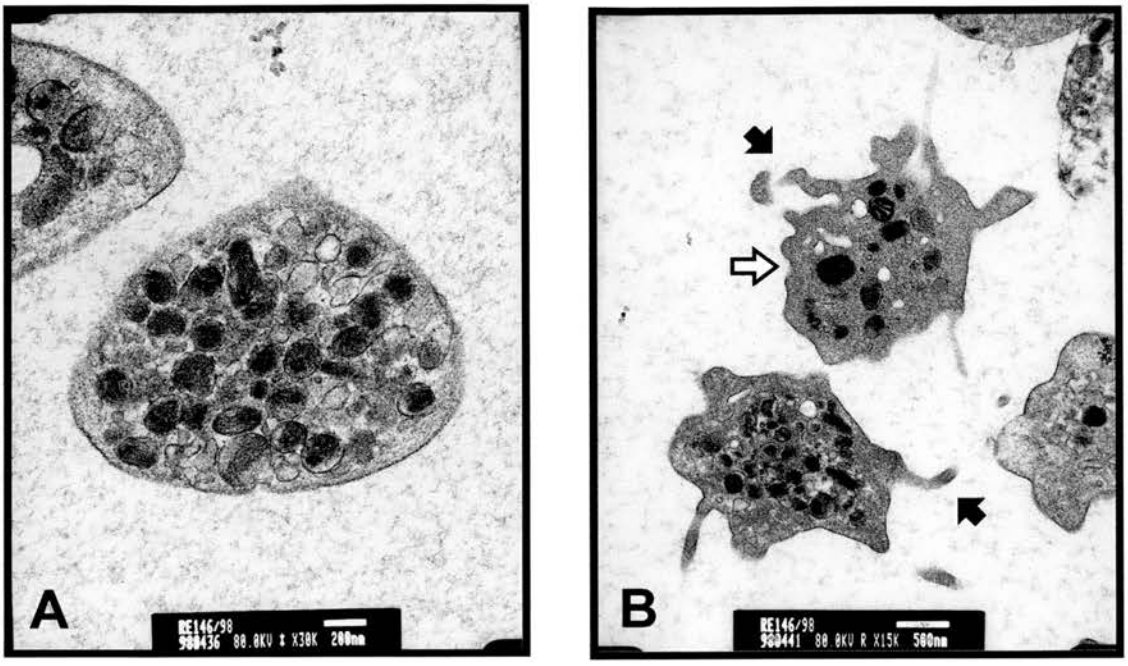


Figure 3.9: Washing of platelets does not induce activation. Transmission electron micrographs (TEMs) of **(A)** freshly isolated washed platelets displaying a smooth unactivated morphology, with an even distribution of alpha and dense granules. **(B)** Freshly isolated ADP activated platelets bearing filopodia extensions (arrows) and evidence of granule coalescence (open arrow) indicative of activation. Scale is as indicated by white bars.

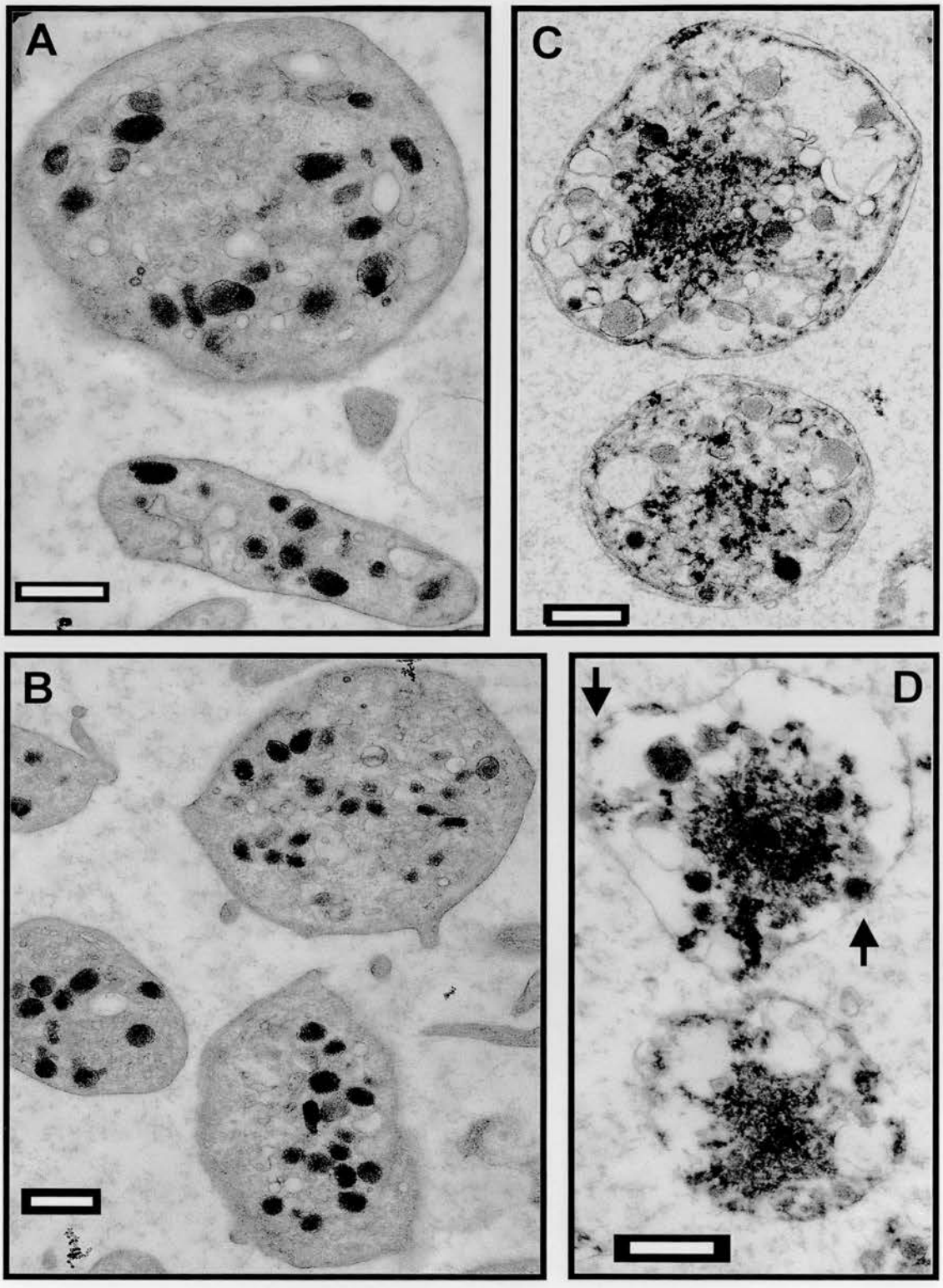


Figure 3.10: **Aged platelets exhibit cytoplasmic condensation.** Transmission electron micrographs of platelet preparations. Washed platelets fixed immediately following isolation **(A)**; aged platelets maintained in citrated plasma for 24 h **(B)**; and washed platelets maintained in HBSS for 12 h **(C)** and 24 h **(D)**. Note evidence of granule fusion with the plasma membrane (arrows). The white bar scale represents 500nm.

3.5 Aged platelets exhibit cell surface changes of apoptosis

In view of the morphological changes suggestive of an apoptosis-like program of platelet death, bearing similarities to those observed in granulocytes, we went on to seek comparable plasma membrane changes. Although Ca^{2+} dependent exposure of phosphatidylserine (PS) in the outer membrane of platelets was originally demonstrated to be a marker of platelet activation with procoagulant properties (Bevers *et al* 1983), PS exposure is also recognised as a reliable marker of cells undergoing caspase-dependent cell death (Vanags *et al* 1996; Martin *et al* 1995; Koopman *et al* 1994). In granulocytes, PS exposure and caspase activation are tightly linked to nuclear changes typical of apoptosis (Fadeel *et al* 1998; Zhuang *et al* 1998; Knepper-Nicolai *et al* 1998). In addition, we sought to confirm the morphological evidence of granule fusion with the plasma membrane during platelet death.

3.5.1 Aging platelets progressively expose phosphatidylserine at their cell surface

By flow cytometry we determined the level of PS exposure using FITC-conjugated annexin-V, a high affinity probe for PS (Koopman *et al* 1994). Flow cytometric analysis of control platelets aged for 24 h in citrated plasma and labelled with annexin-V-FITC revealed a bimodal distribution with typically no more than 9 ± 1% found positive (Figure 3.11). Although a background level of 0.2-0.5% of fresh platelets bound annexin-V, any increases were not apparent until at least 8 h of culture where annexin-V binding increased steadily to around 9% by 24h, with minimal increases seen thereafter (Figure 3.12). In contrast, washed platelets aged in the absence of plasma, which again contained few annexin-V binding cells in the first 6 h of culture, rapidly switched after 8 h to be >80% positive by 15 ± 2 h (Figure 3.11 & 3.12). To address the confounding possibility that PS-exposure was simply a result of the cell having insufficient energy to maintain the "flippase" activity responsible for keeping membrane asymmetry, washed platelets were aged in the presence of varying concentrations of glucose (1 mg ml^{-1} to 10 mg ml^{-1}). Following assessment of the level of PS-exposure by annexin-V binding and flow cytometry, no significant differences were seen (Figure 3.13). In concordance with this we are not aware of any significant variation between the typical

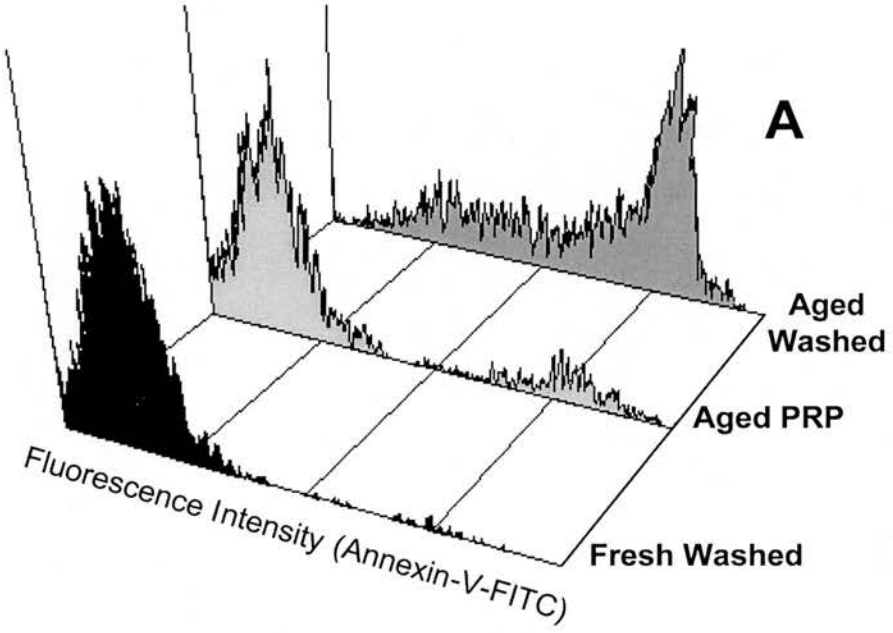


Figure 3.11: Aged platelets express phosphatidylserine at their surface. Fresh washed platelets, or washed platelets aged in HBSS in the absence of serum protein (aged washed), and platelets aged in citrated plasma (aged PRP), 24 h, were assessed by flow cytometry for phosphatidylserine exposure using annexin-V-FITC.

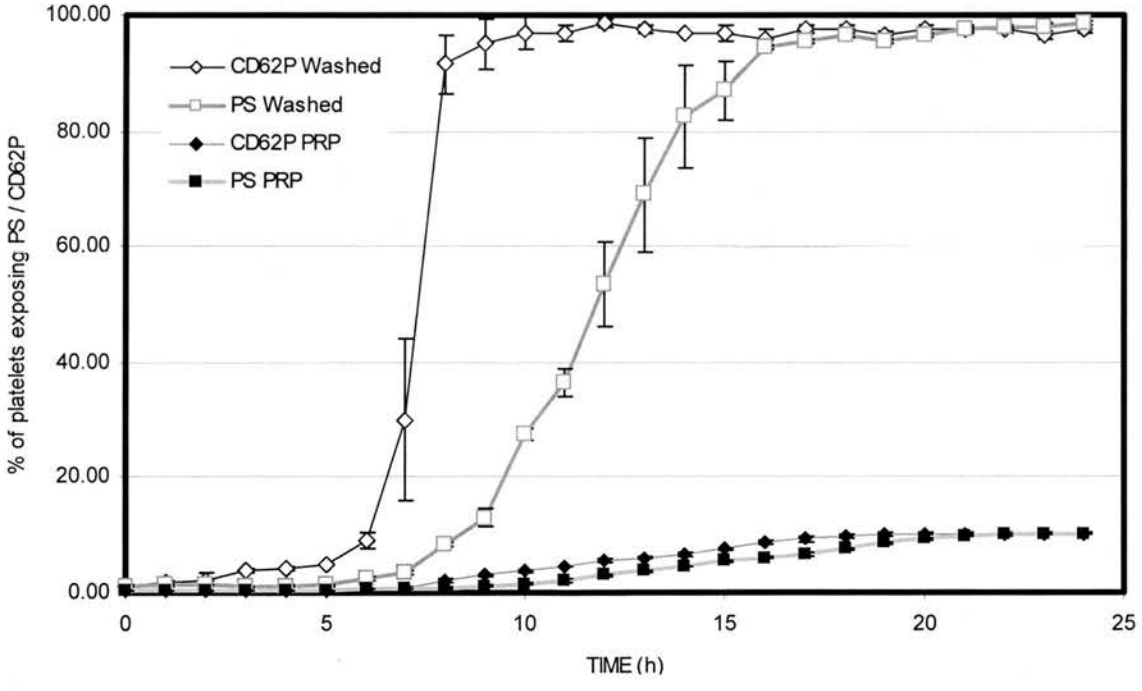


Figure 3.12: Platelets slowly and progressively expose PS and CD62P at their cell surface. Platelets were monitored by flow cytometry every hour for cell surface changes, as previously described. Open points represent washed platelets, whilst closed points represent platelets aged in PRP. PS exposure is denoted by squares, and CD62P by diamonds. Data represent means ± one S.D of two determinations

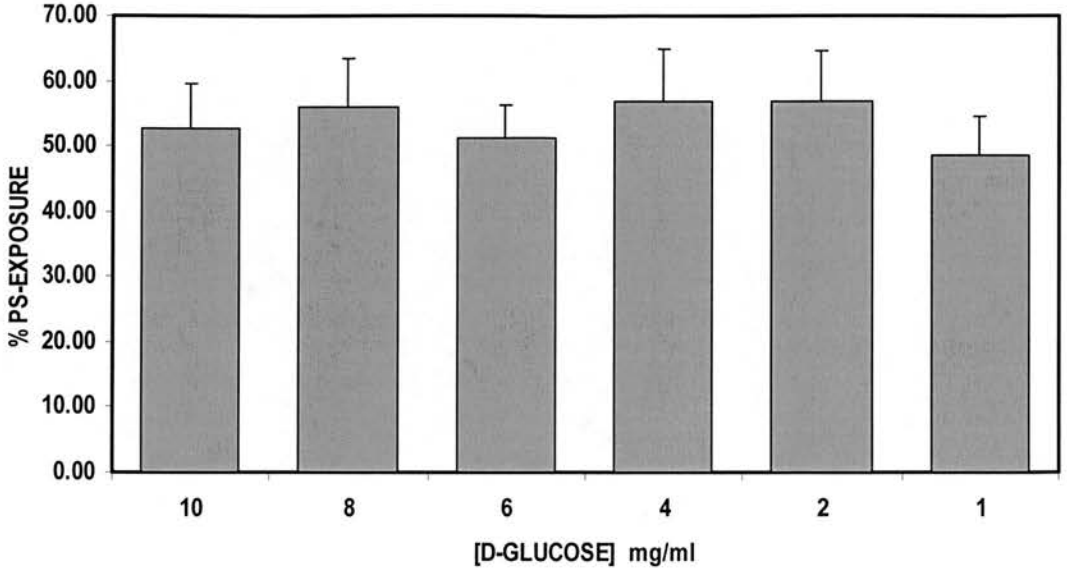


Figure 3.13: Glucose concentration has no effect on the level of PS-exposure. Fresh washed platelets were aged in the presence of varying levels of d-glucose, and the percentage of cells exposing PS quantified by flow cytometry as described previously. The data represents mean ± one S.D. of n = 4. No significant changes are witnessed.

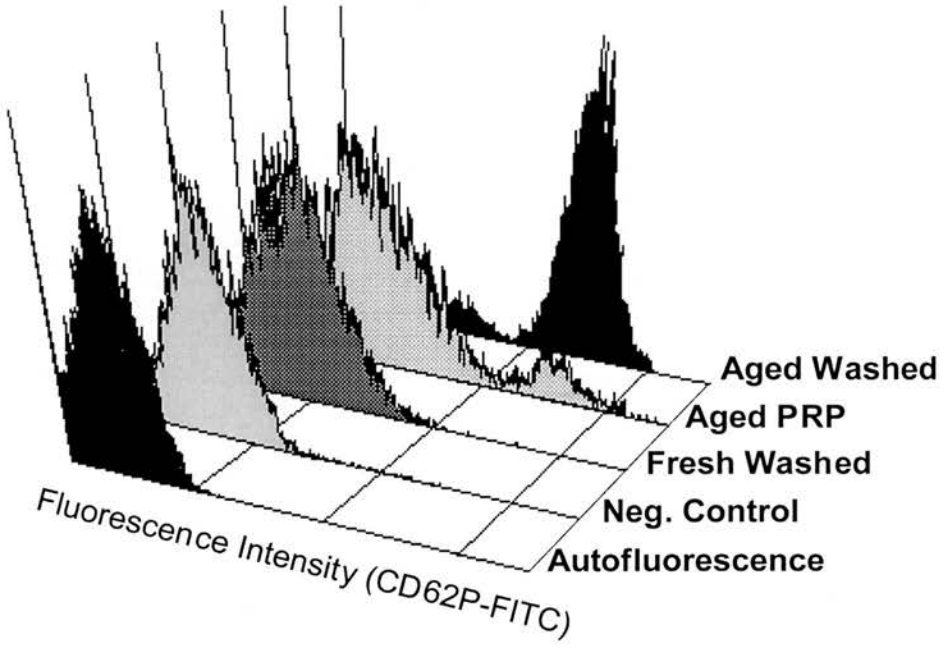


Figure 3.14: Aged platelets express P-selectin at their surface. Fresh washed platelets, or platelets aged in HBSS in the absence of serum protein (aged washed), and platelets aged in citrated plasma (aged PRP), 24 h, were assessed by flow cytometry for granule cell fusion with the plasma membrane by monitoring for cell surface P-selectin expression using a FITC-conjugated anti-P-selectin mAb. The FL1-H channel was set to the autofluorescence of unlabelled platelets and confirmed that non-specific binding of control antibodies was minimal.

glucose concentrations in HBSS of 1 mg ml^{-1} to normal clinical biochemistry values for plasma glucose of 0.7-1.2 mg ml^{-1} (Kumar and Clark 1998).

3.5.2 Aging platelets progressively expose CD62P at their cell surface

To confirm the possibility of granule fusion with the cell membrane we probed for the intracellular alpha granule marker P-selectin (Israels *et al* 1992)(Figure 3.14). Reassuringly we found that platelets maintained in citrated plasma did not express any cell surface P-selectin in the first 8 h of culture (Figure 3.12), again evidence against platelet activation due to the washing procedure. Indeed, after aging for 24 h in the presence of plasma we found that only a small proportion (~ 10%) of platelets expressed P-selectin. However, washed platelets cultured in HBSS in the absence of serum rapidly mobilised intracellular stores of P-selectin following 6 h of in vitro culture, so that all cells were positive by 7-10 h (Figure 3.12). Given the slow progression of both the PS and CD62P exposure, and the time period between platelet preparation and onset of the changes, especially in light of the TEM, we are confident that this profile does not match the acute response typical of activated platelets.

3.5.3 Platelets aged in the presence of calcium expose PS, but undergo microvesiculation

Initial experiments to age platelets had been within their native citrated PRP, and therefore contained very little calcium (~40μM) (Bell *et al* 1990). Due to the clotting factors present, re-addition of calcium to physiological levels results in initiation of the coagulation cascade with clot formation and retraction, a technique routinely used to produce autologous serum (Haslett *et al* 1985*)*. Hence, parallel experiments on aging washed platelets had been conducted in HBSS without calcium. To investigate effects of calcium, platelets were aged following washing and resuspension in either HBSS w/ Ca^{2+}, HBSS w/o Ca^{2+}, platelet poor plasma derived serum (PPPDS), re-citrated PPPDS, or left untouched to age in PRP. A comparable percentage of platelets exposed PS within HBSS w/ or HBSS w/o or PPPDS, with few platelets in PRP becoming PS positive (Figure 3.15). However, flow cytometry forward and side scatter parameters revealed that platelets aged with Ca^{2+}present, in either

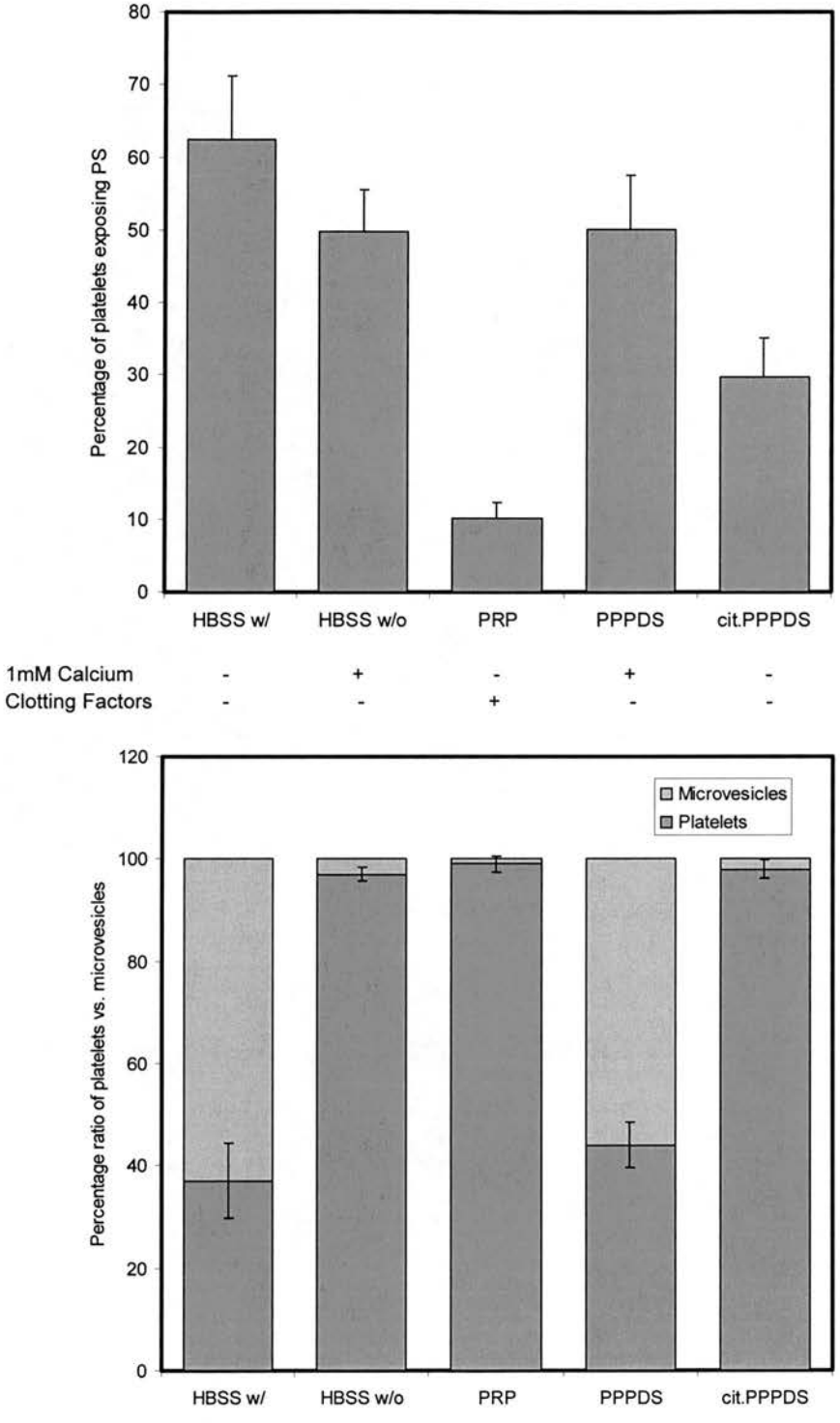


Figure 3.15: Aging platelets with calcium either in the presence or absence of clotting proteins results in microvesiculation and PS-exposure. Fresh platelets were left untreated, or washed and resuspended into HBSS with Ca^{2+}, HBSS without Ca^{2+}, PPPDS, or re-citrated PPPDS and incubated at 37°C for 16 h. The level of PS exposure and microvesiculation was assayed by flow cytometry. Data represent mean ± one S.D. of n = 3.

HBSS w/ or PPPDS, had undergone microvesiculation, with evidence of platelet loss when compared to conditions without calcium present (Figure 3.15). Comparison between both HBSS conditions indicated a small but significant ($p<0.05$) increase in the level of PS exposure from 50 ± 5 % to 63 ± 8 % in the presence of calcium. Given the microvesiculation that occurred with calcium present, a marker indicative of platelet activation, this increase in PS exposure may reflect an activation-induced phenomenon. Likewise, platelets aged in PPPDS, and therefore with calcium present but with most clotting factors removed, showed similar level of PS exposure and microvesiculation (Figure 3.15). Intriguingly, platelets that had been aged in cit.PPPDS (PPPDS that has been re-citrated, and therefore is without calcium and clotting factors) had not microvesiculated, but did show a significant increase ($p<0.001$) in PS exposure when compared to PRP, 30 ± 5 % and 10 ± 2 % respectively. As the only major difference between platelets aged in PRP, or washed and aged in cit.PPPDS is the removal of clotting factors, the data indicates that a putative plasma derived survival factor was being removed during the coagulation process.

The inability of aged platelets to maintain an asymmetric distribution of PS under Ca^{2+} free conditions was strong evidence of an apoptotic-like constitutive cell death program and not an activation induced phenomenon for two reasons: firstly, activated platelets are well characterised to express a PS translocase that re-establishes an asymmetric distribution, unless platelets have undergone secondary events of aggregation (Bevers *et al* 1989; Bondanza *et al* 2000); secondly, PS exposure on platelets aged in PRP or aged in HBSS w/ o following washing occurs in a calcium free medium, whilst activation-induced PS exposure is well characterised to be a highly calcium dependent event (Comfurius *et* al 1990). Accordingly this suggests that the molecular basis of PS exposure by activated cells differs from that underlying PS exposure by dying cells.

3.6 Aged platelets are selectively cleared by professional and semi-professional phagocytes

In vivo, the most important feature of a cell death program is that intact effete cells are recognised and rapidly ingested by professional and semi-professional phagocytes (Savill *et al* 1989; Savill and Fadok 2000). In keeping with this, we found that fluorescent-labelled platelets cultured for 18 h in the absence of plasma appeared to readily associate with human Mϕ, as evidenced by epi-fluorescence microscopy. However due to the small size of platelets, whether this represented binding or internalisation could not reliably be determined.

3.6.1 Confocal microscopy and TEM confirms phagocyte ingestion of aged platelets

Aged platelets were incubated with 6-day old human Mϕ (30 min) to allow phagocytosis, and cell samples prepared for TEM. As can be seen in the representative electronmicrograph, the Mϕ displays clear evidence of having ingested two aged platelets, both within the cell membrane (Figure 3.16). In addition Bowes melanoma cells were also able to phagocytose aged platelets, again evidenced by TEM (Figure 3.16). Secondary conformation of ingestion, and not surface binding, was obtained with the use of confocal microscopy. Progressive Z-axis scans confirmed that fluorescent-labelled aged platelets were dispersed throughout the cell within phagolysosomal vacuoles, strongly arguing against the possibility of platelets being lodged between the many surface protrusions and invaginations present on the macrophage surface (Figure 3.17).

3.6.2 A novel flow cytometric based method allows quantification of platelet phagocytosis

In order to quantify platelet ingestion by a range of phagocytes we developed a novel flow cytometric method that was dependent on incubating the phagocytes with platelets that had been pre-labelled with an orange fluorescing dye (CM-Orange) (Figure 3.18). Phagocytes were then analysed by flow cytometry where they were readily resolved from platelets by forward and side scatter, and therefore any shift in orange fluorescence of the phagocyte population could be attributed to

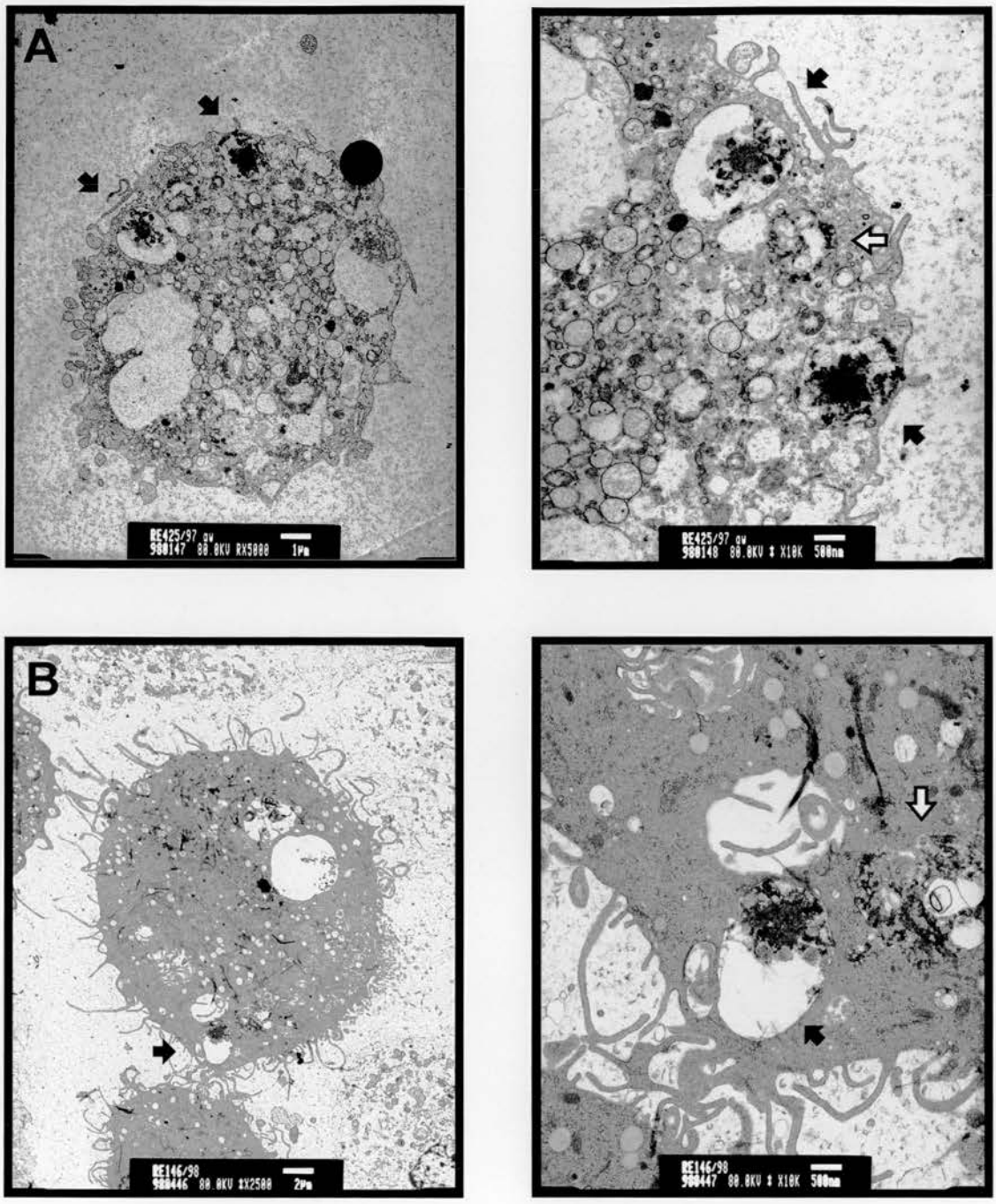


Figure 3.16: Phagocytes readily ingest aged platelets. Representative TEMs showing cytoplasmic regions just beneath the plasma membrane of a human monocyte derived macrophage **(A)** and a Bowes melanoma cell **(B),** both containing distinct evidence of having ingested aged platelets (solid arrows), and possibly for digestion of other aged platelets (open arrows). The scales are as indicated on white bar.

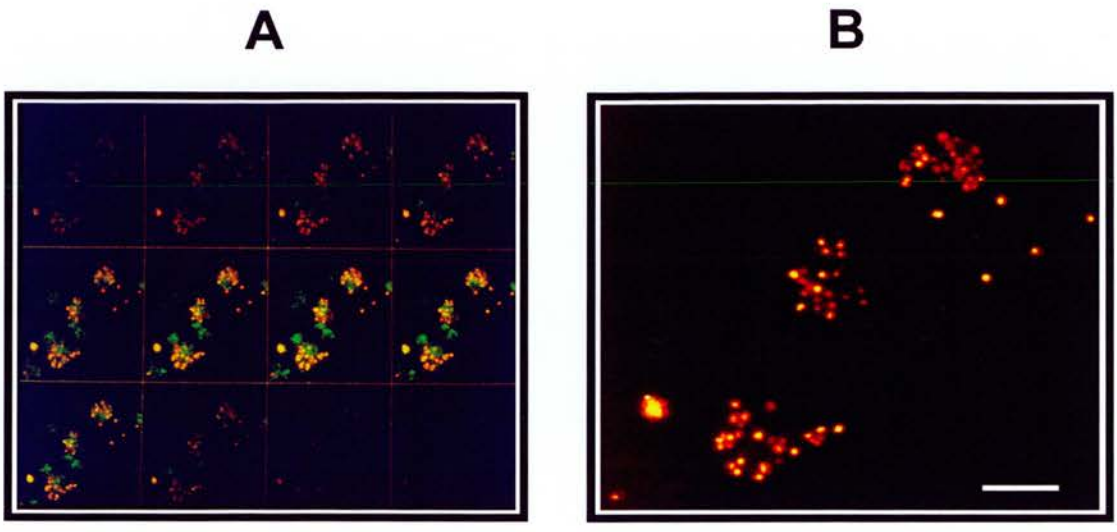


Figure 3.17: Aged platelets are ingested by phagocytes. Scanning confocal microscopic images of human MΦs having ingested fluorescent labelled aged platelets. **(A)** Progressive Z-axis scans through the cell revealed platelets to be dispersed throughout the inside of the MΦs and to not be surface bound, thus supporting internalisation. **(B)** Reconstruction of the focal planes reveal many aged platelets (>20) to have been ingested by each macrophage. The white scale bar represents 10μm.

interacting platelets (Figure 3.18). To discriminate between adherence and ingestion we labelled the phagocytes prior to flow cytometry with an anti-CD61-FITC mAb. Also known as glycoprotein IIIa (GpIIIa), CD61 is a platelet specific cell surface marker, whose expression was not found to alter as platelets were aged in culture, in comparison to freshly isolated platelets (Figure 3.18). Results could also be reproduced with other platelet specific markers such as CD42a or CD41. Reassuringly, we observed that anti-CD61 mAb routinely failed to label our phagocyte populations suggesting that platelets were ingested, in keeping with TEM, and that any adherent platelets were removed prior to flow cytometry during trypsin/EDTA treatment to lift the adherent macrophages. Assessment of cytospin preparations by confocal microscopy in parallel to cytometry confirmed that the two methods closely correlated, proving the efficacy of the system. In contrast, and as a control comparison, we confirmed that freshly isolated labelled platelets adhered to the surface of freshly isolated monocytes (Figure 3.18), a conclusion reported by others (Rinder *et al* 1991).

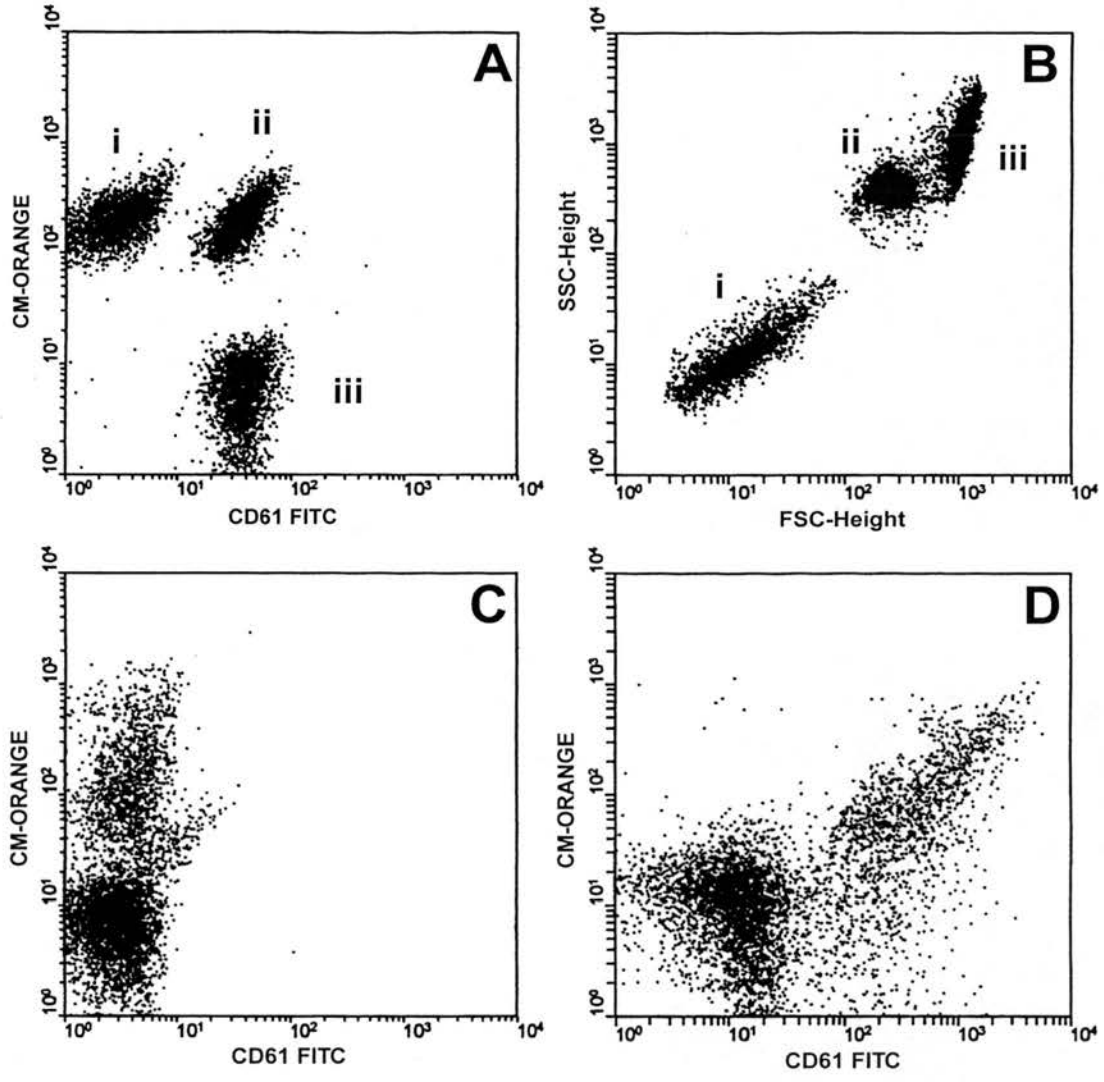


Figure 3.18: Ingestion of aged platelets is readily quantified with a flow cytometric method. (A) Human platelets, pre-labelled with CM-Orange **(i)** and aged in HBSS in the absence of plasma, were confirmed to express the platelet specific marker CD61 with an FITC-conjugated mAb **(ii)**, whose expression remained unaltered relative to freshly isolated CM-Orange unlabelled platelets **(iii)**. **(B)** CM-Orange labelled platelets **(i)** incubated with human macrophages **(ii)** or with Bowes melanoma cells **(iii)** for times indicated elsewhere, were readily resolved from the phagocytes according to their forward and side scatter properties. **(C)** CM-Orange-labelled platelets that associated with the phagocyte population are seen as a distinct subpopulation that is shifted in orange fluorescence. These platelets were predominantly ingested given that they failed to dual label with the CD61 mAb. Similar results were found with mAbs to other platelet markers such as CD41 or CD42a. **(D)** In contrast, freshly isolated monocytes readily bind, but do not ingest, freshly isolated CM-Orange labelled platelets, evidenced by a shift in both channels.

3.6.3 Aged platelets are phagocytosed by a range of cell lines tested

Although fresh and activated platelets on microscopic examination were observed to adhere to all phagocytic cell lines tested, only aged platelets were found to be ingested following flow cytometric analysis (Table 3.1). We also observed that the degree of phagocytosis was always greater for platelets aged in the absence of plasma survival factors than those aged in citrated plasma. Typically, after a 30 min interaction, greater than 85 ± 11% of human Mϕ and around 65 ± 7% of Bowes melanoma cells ingested platelets aged in the absence of plasma (Table 3.1), compared to only 30 ± 12% and 20 ± 6% respectively when aged in the presence of citrated plasma. Moreover, time-course experiments repeatedly showed that in a fixed-time phagocytosis assay the maximal level of ingestion by human Mϕ and Bowes melanoma cells was achieved with platelets aged for 12 h in the absence of plasma or 24 h for those cultured in citrated plasma.

Feed	Phagocyte			
	MDMs	BOWES	TEPM	HUVEC
Fresh platelets	3 ± 2	2 ± 1	1 ± 1	2 ± 1
Aged washed platelets	85 ± 11	65 ± 7	40 ± 8	47 ± 5
Activated platelets	3 ± 1	2 ± 1	3 ± 2	1 ± 2

Table 3.1: **Aged platelets are phagocytosed.** Fresh, aged washed, and activated platelets were incubated with a panel of phagocytic cells; human MDMs, human Bowes melanoma cells (BOWES), murine thioglycollate-elicited peritoneal macrophages (TEPM), and human umbilical vein endothelial cells (HUVEC). Phagocytosis was assayed by flow cytometry as described previously. Data represent mean ± S.D. from a minimum of at least four experiments

3.6.4 Ingestion of aged platelets by phagocytes occurs specifically via scavenger receptors

The degree to which cell lines ingested aged platelets, whether cultured in the presence or absence of plasma, was found to be unaffected by various well-characterised inhibitors of recognition. (Table 3.2) (Hart *et al* 1997). These included the integrin inhibitor RGDS, the PS receptor-competitor phospho-L-serine, the cationic sugars glucosamine and galactosamine, the anti-CD36 mAb SMϕ, and the anti-CD14 mAb 61D3 (Table 3.2). However, greater than 82 ± 10% inhibition was observed with fucoidan, a recognised inhibitor of the scavenger receptor pathway, in contrast to the lack of inhibition by dextran at the same concentration, which served as control. Polyinositol, another inhibitor of the scavenger receptor pathway, but not its standard control polycytidine, also inhibited recognition by 38 ± 4%, although not as effectively as fucoidan (Table 3.2). Nevertheless, further confirmation of a major role for the scavenger receptor was obtained with the anti-murine scavenger receptor mAb 2F8, which inhibited mouse peritoneal macrophage uptake of aged platelets by 76 ± 13%.

Since our studies had also shown that aged platelets expressed P-selectin (Figure 3.14), which is known to mediate adhesion of activated platelets to monocytes (de Bruijine-Admiral *et al* 1992), and given that fucoidan is known to bind the lectin domain of P-selectin, we explored the role of P-selectin in platelet recognition. By using a function-blocking mAb to P-selectin, clone G1/G1-4, which recognises the lectin domain, we found that the recognition of aged platelets by human Mϕ and Bowes melanoma cells was only weakly affected (14 ± 3 %), and no synergy with polyinositol was observed (12 ± 4%) (Table 3.2). This suggests that although P-selectin had a minor role in the phagocytosis of aged platelets, potentially mediating an initial tethering, it was not primarily responsible for mediating phagocytic recognition and clearance, a conclusion in agreement with others (Michelson *et al* 1996; Berger *et al* 1998).

Table 3.2: Platelet ingestion is mediated by the scavenger receptor.

Platelets aged with or without plasma	Concentration	Mϕ		BOW ES		TEPM	HUVECS
		-	+	-	+	-	-
EGTA	1 mM	-	nd	-	nd	nd	nd
RGDS	1 mM	-	-	-	-	-	-
RGES	1 mM	-	-	-	-	-	-
Phospho-L-serine	1 mM	-	-	-	-	-	-
N-acetyl Glucosamine	20 mM	-	-	-	-	-	-
N-acetyl Galactosamine	20 mM	-	-	-	-	-	-
Poly-Inositol	100 µg/mL	++	++	++	++	++	++
Poly-Cytidine	100 µg/mL	-	-	-	-	-	-
Fucoidan	100 µg/mL	+++	+++	+++	+++	+++	+++
Dextran	100 µg/mL	-	nd	-	nd	nd	nd
mAb 2F8 (anti-scavenger receptor)	10 µg/mL	na	na	na	na	++	na
mAb G-1 (P-selectin blocking Ab)	10 µg/mL	+	nd	+	nd	nd	nd
mAb G-1 + 100 ug/mL poly-inositol	10 µg/mL	++	nd	++	nd	nd	nd
mAb G-1 + 1mM EGTA	10 µg/mL	+	nd	+	nd	nd	nd
mAb CRC81 (anti-P-selectin)	10 µg/mL	-	nd	-	nd	nd	nd

The level of inhibition, compared to control, by the above compounds on the ingestion of platelets that had been aged in either the absence or presence of plasma protein were assessed by flow cytometry as follows: -, 0-5% inhibition, not significant; +, 5-30% inhibition*; ++, 30- 60% inhibition**, +++, 60-100% inhibition***. na, not applicable. nd, not determined. Data represent $n = 5$, * $p < 0.05$, **·*** $p < 0.01$.

3.6.5 A chymotrypsin sensitive protein on the aged platelet is required for phagocytosis

Previous work on the *in vivo* clearance of platelets has proposed few potential molecules and mechanisms involved, with reoccurring data spanning over three decades. These include the loss of sialic acid residues (Greenberg *et al* 1975; 1979), and the involvement of the Fc receptor FcRIIA (McKenzie *et al* 1999), all leading to a 'recognition competent' platelet and its clearance. However, the majority of studies, with the exception of the Fc data, promote the idea that platelets are passive in the process and do not direct these changes, and that they occur to them through 'attrition' over their lifespan. As a brief investigation into the potential platelet molecule mediating clearance in our system, aged washed platelets were treated with a panel of proteases, and a sialidase at low concentrations (10 $\mu g\ ml^{-1}$), before washing, incubation with MDMs, and assessment of phagocytosis by flow cytometry. These included the serine proteases chymotrypsin and elastase, protease nine and fourteen, and the thiol protease papain. In addition the sialidase neuraminidase, which is able to specifically cleave sialic acid residues from glycoprotein conjugates, was also tested. Under the enzyme concentrations stated in materials and methods chymotrypsin and papain significantly inhibited phagocytosis by 65 ± 8 % and 23 ± 5 % respectively (Figure 3.19). In addition, data against general background surface protein cleavage, due to high protease concentrations, was evidenced by doubling the enzyme amount. At this higher concentration only chymotrypsin gave a significant increase in the level of inhibition of phagocytosis, 81 ± 4 % ($p < 0.05$), suggesting that at both of these relatively low protease concentrations the target protein removed must contain an exposed highly chymotrypsin sensitive residue. Chymotrypsin has been shown to require aromatic or non-bulky polar side chains on the amino side of the scissile bond (Stryer 1998). Although the data is insufficient to identify a specific molecule it does suggest that a platelet surface protein, which may bear modified sugars such as sialic acid, is required for clearance.

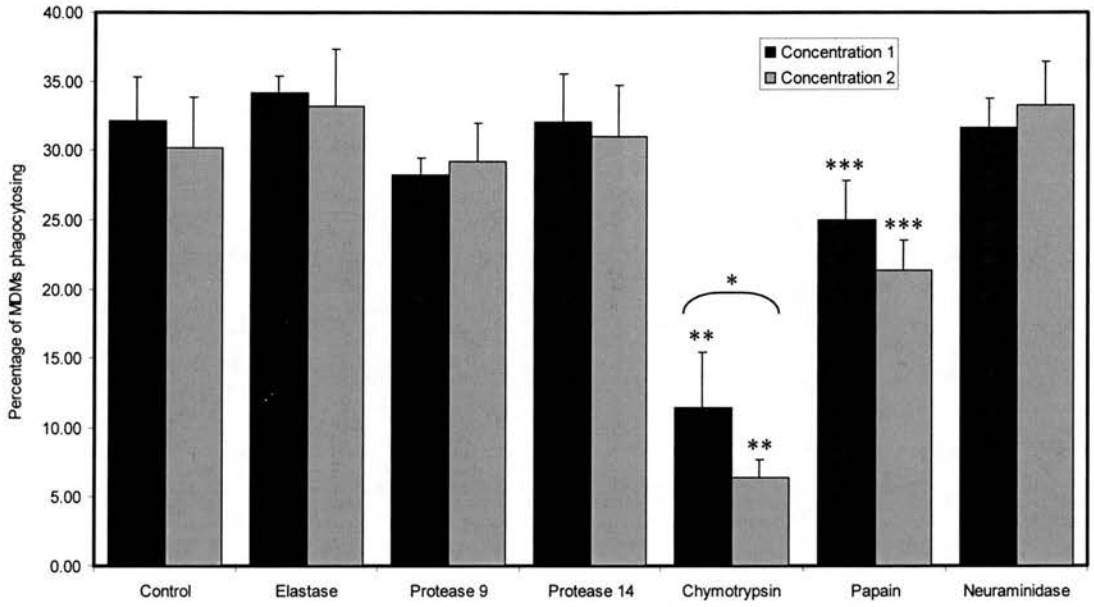


Figure 3.19: Pre-treatment of aged washed platelets with the serine protease chymotrypsin inhibits phagocytosis. Aged platelets were pre-treated with a panel of proteases and a sialidase, washed, and then incubated with human MDMs. Phagocytosis was quantified by flow cytometry as previously described. The grey bars represent a doubling of the enzyme concentration used in the experiments represented by the black bars. A significant reduction in the level of phagocytosis compared to control occurred on platelet treatment with chymotrypsin ($p < 0.001$,**), and to a lesser extent with papain ($p < 0.02$, ***). Interestingly, only chymotrypsin treatment inhibited phagocytosis to a greater extent on increasing concentration ($p < 0.05$, *). Data represent ± one S.D. of $n = 3$

Discussion

Platelets play a crucial role in haemostasis and thrombosis, with the consequence that they are of central importance in common disorders such as myocardial infarction and stroke. However, little has been known of any candidate mechanisms for safe clearance of these anucleated blood cells every day. Prompted by earlier work on constitutive apoptosis in other key blood cells, granulocytes, we sought evidence for any constitutive death program within platelets. Key findings of this study include the discovery that platelets expressed members of the Bcl-2 family of cell death-regulating proteins, in keeping with earlier work (Vanags *et al* 1997), and that there was an apparent pro-apoptotic shift observed in platelets aged for 18 h in citrated plasma. We also observed that aged platelets lost the ability to aggregate in response to agonists such as ADP, and failed to adhere and spread on the key subendothelial matrix component collagen. Given that serum is a rich source of survival factors for granulocytes, we reasoned that the physiological milieu of platelets, plasma, was likely to also contain factors capable of retarding their cell death. We therefore sought evidence of accelerated cell death when platelets were cultured in the absence of plasma, finding that they not only exhibited an accelerated loss of function but also displayed many features in common with programmed death of granulocytes. Such changes included morphological evidence of cytoplasmic condensation and cell surface expression of the "eat me" signal phosphatidylserine (Fadok *et al* 1992), and granule components such as P-selectin. Importantly, however, there was strong evidence that such aged platelets retained plasma membrane integrity since there was no detectable release of the cytoplasmic marker enzyme lactate dehydrogenase, and nor did the aged cells admit actin-binding phalloidin-FITC, used as a novel "vital" dye. Furthermore, aged platelets were recognised and ingested by all professional and semi-professional phagocytes tested, and by a mechanism in which scavenger receptors predominated.

The abrogation of platelet function witnessed on aging represents a strong parallel to granulocyte apoptosis. Both cell types can mediate responses potentially deleterious to a metazoan, with granulocytes (and platelets to a lesser extent) able to secrete a plethora of pro-inflammatory mediators, and platelets able to form thrombi. Thus, down regulation of these functions at the onset of

cell death acts to safeguard against any uncontrolled and adverse consequences (Whyte *et al* 1993). What actually mediates loss of the platelets ability to aggregate has not been elucidated. However, although the level of CD41 or CD61 does not alter on platelet aging, the possibility exists that the complex dissociates, or inside-out integrin signalling is blocked, hence resulting in effective loss of the fibrinogen receptor and an inability of platelets to aggregate. This dissociation would be an elegant way of effectively "switching off" any potential for unwanted thrombi formation, and the molecular mechanisms responsible for such an event would be an excellent target for future antithrombotic drug design.

The PS exposure witnessed on aging is unusual in platelets for two reasons. Firstly, PS exposure is a classical marker of platelet activation that helps to provide a procoagulant surface that accelerates thrombin generation. This exposure is well characterised to be a highly Ca^{2+} dependent process, but in our system platelets are aged in either ~45μm Ca^{2+} in the PRP, or no Ca^{2+} when aged in the HBSS. Both result in PS exposure, with Ca^{2+} free conditions producing a greater level of PS exposure. These differences between the levels of PS exposure are not thought to reflect the level of Ca^{2+}, but rather the presence of the native plasma and hence putative survival factors. In addition, this change occurs slowly and progressively over an 8 h timecourse, which is at odds with a more typical acute exposure of PS seen upon platelet activation. Secondly, and in contrast, aging platelets in levels of physiological Ca^{2+} (~1mM) results in high levels of PS exposure whether plasma factors are present or not, and a large amount of microvesiculation occurs that is not present without Ca^{2+}. This form of PS exposure appears to be quite distinct from that occurring without Ca^{2+} and is more indicative of an activation-dependent event. In this respect it is also quite clear from our work, and that borne out by many others, that activated platelets are not cleared from the circulation or phagocytosed *in vitro* or *in vivo* (Michelson *et al* 1996; Berger *et al* 1998), in contrast to the senescent platelets which in our system are readily phagocytosed.

The exposure of PS by an aged platelet may seem a little counter intuitive given its powerful procoagulant properties. However, a low level of PS exposure alone is relatively benign given that its main role is in accelerating the generation of thrombin and hence requires tissue factor, exposed on

subendothelial matrices after endothelial damage, to effect a response. In this respect an aged platelet encountering such a site would simply be "pre-primed" to aid in aggregation. Nevertheless, PS redistribution during cell death is a highly conserved and ubiquitous event, and has been strongly implicated in phagocytic mechanisms (Fadok *et al* 1992). Although we have implicated a scavenger receptor mechanism in the clearance of aged platelets, our lack of effect when using the reagent phospho-L-serine does not rule out the involvement of PS in phagocytosis. Firstly, phospho-L-serine is not a particularly good competitive inhibitor of PS. Secondly, given the short size of PS within the relative "forest" of the plasma membrane glycocalyx of platelets, we believe PS is more likely to function as an ancillary molecule to an initial scavenger receptor interaction, acting like "Velcro" to mediate a tight interaction. The evolutionary origin of PS exposure by a dying cell represents an elegant and simple "external tag of death". As living functional cells posses an active translocase to maintain PS on the inner side of the membrane, any form of death stimulus or cell death occurrence could be argued to eventually result in loss of PS asymmetry due to the intrinsic membrane flip-flop of phospholipids. In this respect "inert" microvesicles containing a PS component have been demonstrated to be phagocytosed by macrophages (Rimle *et al* 1984).

It will also be important to define in more detail the molecular mechanisms by which phagocyte class-A scavenger receptors mediate recognition and ingestion of aged platelets, as clearly indicated by the inhibitor studies presented. Although the role of the scavenger receptor has already been implicated in the clearance of apoptotic thymocytes by mouse macrophages (Platt *et al* 1996), the ligands displayed by dying cells that lead to their recognition by scavenger receptors are currently less well known. Furthermore, the novel finding that cells of endothelial origin can phagocytose platelets opens a new insight into potential sites of platelet removal. As discussed in the introduction most work has emphasised the spleen and liver as the primary sites of platelet removal, as implicated by radioactive tracer studies. However, if endothelial cells lining the vasculature *in vivo* are capable of recognising and clearing effete platelets this would not have been detected by the tracer experiments, as there would have been no "concentrated" site of radiation. The phagocytosis of aged platelets also has implications for the maintenance of normal levels of circulating platelets - thrombostasis. Although of limited *in vivo* relevance, this data suggests the potential that diseases of

circulating platelet numbers may reflect abnormalities or defects in the cell death program leading to recognition, or alternatively in the phagocytosis step itself, resulting in either increased or decreased platelet removal from the circulation. Given the implication of the scavenger receptor in mediating aged platelet uptake, and its known function as a receptor for acetylated lipoproteins (Traber *et al* 1993), it may be interesting to examine whether familial hypercholesterolemics, or similar conditions resulting in raised serum lipoproteins, effectively "self-inhibit" uptake of effete platelets.

Since constitutive death in plasma-deprived platelets cannot be assessed for typical nuclear changes, since these cells have no nucleus, we feel it is not appropriate to label this form of cell death as apoptosis, which by semantic definition requires nuclear condensation and oligonucleosomal DNA cleavage. However, we believe that the data indicates that platelets can undergo a form of cell death that can be regulated by exogenous influences, in particular plasma-derived survival factors. In keeping with this, we observed that the return of plasma-deprived platelets to plasma slowed the phenomena of cell death, most notably returning the rate of loss of aggregation and levels of PS exposure to that observed for platelets cultured in plasma. Ongoing work is directed at the biochemical characterisation of the survival activity present in plasma since a range of candidate cytokine survival factors could not substitute for plasma. Nevertheless it should be emphasised that plasma deprivation appeared merely to accelerate a constitutive death program that was already active in platelets at 37°C and evident after 18-24 h of culture in native plasma. However, study of constitutive death in plasma-replete platelets will be difficult as our preliminary work demonstrated progressive loss of platelets from populations cultured for >24 h, presumably reflecting relatively rapid secondary necrosis of that proportion of cultured platelets undergoing cell death each day.

In conclusion, we have provided *in vitro* evidence that human platelets can undergo a constitutive program of cell death that resulted in the specific recognition of effete cells by phagocytes employing the scavenger receptor as a recognition mechanism. While these findings have potentially important implications for understanding platelet kinetics and the related pathogenesis of thrombotic and bleeding disorders, no firm conclusions on *in vivo* relevance can be drawn from the current data.

Nevertheless, these data raise the exciting prospect that platelet lifespan and clearance is amenable to exogenous regulation for therapeutic purposes.

Chapter 4 – Caspase-Independent Platelet Death

Introduction

Caspases have been the subject of intense investigation since their discovery and the elucidation of their role as the central effectors of the apoptotic cell death program (Thornberry *et al* 1992). Existing as latent proforms within the cytoplasm of most cells, their activation occurs in a hierarchical cascade by cleavage of downstream "effector" caspases by "initiator" caspases, activated in response to a number of initiating stimuli and factors, often promoting oligomerisation (Thornberry and Lazebnik 1998). Having an extremely specific substrate cleavage preference, which also occurs within the proform as the cleavage site for activation, their structure enables them to lie dormant during normal cellular physiology, but to activate with great speed and to large effect when required. One such caspase cascade occurs in response to the so-called "intrinsic" death pathway, named to reflect an internal origin of the decision by the cell to die. Although the initiating biochemical events are yet to be fully understood, the pathway typically proceeds through an increase in the proapoptotic mitochondrial interacting Bcl-2 family members, which mediate mitochondrial perturbations resulting in a loss of the transmembrane potential and an associated release of cytochrome-C (Martinou and Green 2001; Zamzami and Kroemer 2001). The presence of this normally mitochondria exclusive protein in the cytoplasm initiates an irreversible chain of events, ultimately resulting in the cells demise. The initiator caspase-9 forms a cytochrome-C dependent complex with APAF-1, named the apoptosome, and with dATP as a cofactor results in the self-processing of caspase-9 to its active form (Liu *et al* 1996). This in turn activates the effector caspase-3, itself able to activate more caspase-9 resulting in amplification of the cascade, and the caspase-3 mediated cleavage of distinct cellular substrates. These specific directed cleavages, for example nuclear lamins, inactivation of DNA repair enzymes, cytoskeletal proteins, and the direction of cell surface changes, lead to many of the classical changes witnessed during apoptotic cell death, such as nuclear condensation, cell shrinkage, and the specific recognition and clearance by phagocytes.

Given that platelets undergo a constitutive death program without any apparent external stimuli required to initiate it, it could be proposed to be an intrinsic type cell death. Indeed, many of the changes witnessed bear similarities to intrinsic granulocyte apoptosis, including up regulation of

proapoptotic Bcl-2 family members, surface PS exposure, granule fusion with the cell membrane, and clearance. Given the central role of caspases in granulocyte apoptosis, and the mitochondrial / apoptosome pathway in initiating the activation, we investigated what role caspases might play in directing the platelet changes reported in the previous chapter.

Using the poly-caspase inhibitor zVAD-fmk we were unable to alter or attenuate the changes witnessed on platelet death, such as PS exposure, loss of aggregatory response, or the "edibility" of the platelets to phagocytes. In addition, despite containing amounts of caspase-3 comparable to Jurkat cells, we could find no evidence of enzyme activation within the aged platelets by Western-blot analysis or fluorescent substrate cleavage. Interestingly, a more detailed examination of the intrinsic pathway revealed that upon aging platelets underwent mitochondrial permeability transition and released mitochondrial cytochrome-C into the cytoplasm, but unlike apoptotic Jurkats that proceeded to activate caspase-3 as expected, platelets did not. This observation was recapitulated in a cell free system by adding exogenous cytochrome-C and dATP to Jurkat and platelet lysates, whereby Jurkat lysates processed caspase-3 to its active form implying formation of an active apoptosome, whilst platelet lysates did not. Western-blot analysis for the apoptosome components revealed APAF-1 to be present within platelets, but analysis for caspase-9 did not detect this key enzyme.

Despite a previous report claiming caspase-9 to be present within platelets, an extended analysis with both polyclonal and monoclonal antibodies failed to detect the enzyme. However, analysis under less stringent blotting conditions revealed a protein band around the weight of caspase-9, which by two-dimensional electrophoresis was shown to possess a pI inconsistent with it being caspase-9. Direct addition of human recombinant caspase-9 into platelet lysates enabled the cytochrome-C dependent activation of caspase-3 to occur, thus definitively demonstrating the absence of caspase-9 from platelets to prevent cytochrome-C mediated activation of caspase-3, either in a cell free system or within platelets during aging. Intriguingly, the progenitors of platelets, the MKs, contained quantities of caspase-9 comparable to Jurkats, suggesting sequestration, detainment or consumption of the enzyme during platelet formation.

4.1 Platelet death is caspase and calpain independent

Activation of caspases and calpains is reported to be upstream of plasma membrane changes associated with apoptosis, including PS exposure (Martin *et al* 1996; Zhuang *et al* 1998; Knepper-Nicolai *et al* 1998; Vanags *et al* 1996; Naito *et al* 1997), and has been shown to be the most ubiquitous of all apoptotic machinery. Caspase-3 represents a major effector of cellular death programs in the majority of cell systems, responsible for mediating the typical morphological changes witnessed on death (Thornberry and Lazebnik 1998). Activation due to proteolysis by upstream initiator caspases result in the formation of a 17/12 kDa caspase-3 heterodimer, which further dimerises to form an active tetramer with a preferred substrate cleavage consensus DEVD. The stringent requirement of the caspases catalytic specificity is for aspartic acid in the P_1 subsite, with variation within the P_4 site shown to be an important determinant of specificity between caspase family members, which enabled the development of a panel of novel inhibitors (Thornberry *et al* 1997).

4.1.1 PS-exposure by aged platelets is caspase and calpain independent

Using the caspase-3 inhibitor zDEVD-fmk, the poly-caspase inhibitor zVAD-fmk, and in addition the calpain inhibitors ALLN and calpeptin, we looked to see if the level of PS exposure by aged platelets could be modulated. Washed platelets were cultured for 18 h in the presence of the inhibitors, followed by analysis of PS-exposure by flow cytometry using annexin-V-FITC. None of the inhibitors produced any significant effect suggesting no apparent role for caspases or calpains in mediating the aged-induced PS-exposure (Table 4.1). Additionally, and to confirm the role of these proteases in mediating earlier occurring physiological changes we had identified, we also found that the inhibitors had no effect on the refractiveness of aged platelets to the early loss of ADP-induced aggregation (Table 4.2), whether cultured either in the presence or absence of plasma.

Condition / Inhibitor	Concentration	PS-positive washed platelets (after 18 h)	n
	μm	%	
Aged Washed		71.2 ± 6.3	7
DMSO Control	[0.1%]	70.6 ± 3.8	7
ZVAD-fmk	100	84.2 ± 8.0	5
zDEVD-fmk	10	76.0 ± 3.5	5
ALLN	100	71.8 ± 5.9	7
Calpeptin	100	77.3 ± 9.9	5

Table 4.1: Caspase or calpain inhibitors do not modulate PS exposure by washed platelets. Washed platelets were aged for 18 h and analysed for the level of PS exposure by flow cytometry. Data represent mean ± one S.D. n = number of separate determinations, as indicated.

Condition / Inhibitor	Maximum level of aggregation (%)	
	Presence of plasma (24h)	Absence of plasma (2h)
Control	51 ± 9	6 ± 1
zVAD-fmk	57 ± 13	5 ± 2
Calpeptin	48 ± 11	2 ± 1

Table 4.2: Caspase and calpain inhibitors do not modulate the loss of aggregatory response to ADP. Fresh or washed platelets were incubated for times as indicated, and analysed for the level of aggregation following ADP stimulation. Data represent mean ± one S.D. n = 4.

4.1.2 Caspases do not direct surface changes leading to recognition and clearance

Since PS-exposure did not appear to play a role as a primary recognition molecule during phagocytic clearance of senescent platelets, as discussed earlier in chapter 3, we pursued the possibility that caspases might be responsible for directing other surface changes mediating clearance. Therefore, washed platelets were aged in the presence of caspase inhibitors, washed, and incubated with macrophages before analysis of phagocytosis by flow cytometry (Table 4.3). Similarly, phagocyte recognition of washed platelets aged in the presence of caspase inhibitors was no different from control washed platelets, confirming the unusual caspase-independent nature of the surface changes by effete platelets that lead to phagocytic recognition and clearance.

Condition / Inhibitor	Concentration	Recognition of aged washed platelets	N
	μm	%	
Aged Washed	/	100	4
DMSO Control	[0.1%]	99.2 + 4.7	4
zVAD-fmk	100	117.4 + 17.0	4
zDEVD-fmk	10	99.5 + 7.9	4

Table 4.3: Platelets aged in the presence of caspase inhibitors are phagocytosed at comparable levels to control platelets. Data represent normalised mean ± one S.D.

4.1.3 Aged platelets do not process caspase-3 to an active form by Western-blot analysis

Western-blot analysis confirmed that caspase-3 was present as the 32kDa parent species in platelets at protein levels comparable to Jurkats, as evaluated by equal protein loading. However, and in contrast to apoptotic Jurkats, aged platelets had failed to process caspase-3 to its active fragments (Figure 4.1), but did reveal the presence of several proteolysed species of decreasing molecular weight, just less than the p32 proform band. Interestingly, recent reports have documented a similar caspase-3 degradation pattern, which was shown to be calpain-mediated processing to a non-active species (Wolf *et al* 1999; Chua *et al* 2000; Lankiewicz *et al* 2000). In support of this, platelets aged in the presence of the specific calpain inhibitor calpeptin prevented this proteolytic "nibbling". As discussed earlier neither caspase or calpain inhibitors had any effect on the rate of aging or clearance, therefore it appears this calpain mediated proteolysis is coincidental, is unlikely to be a deliberate step to inactive caspases, and probably reflects general catabolism of cellular proteins.

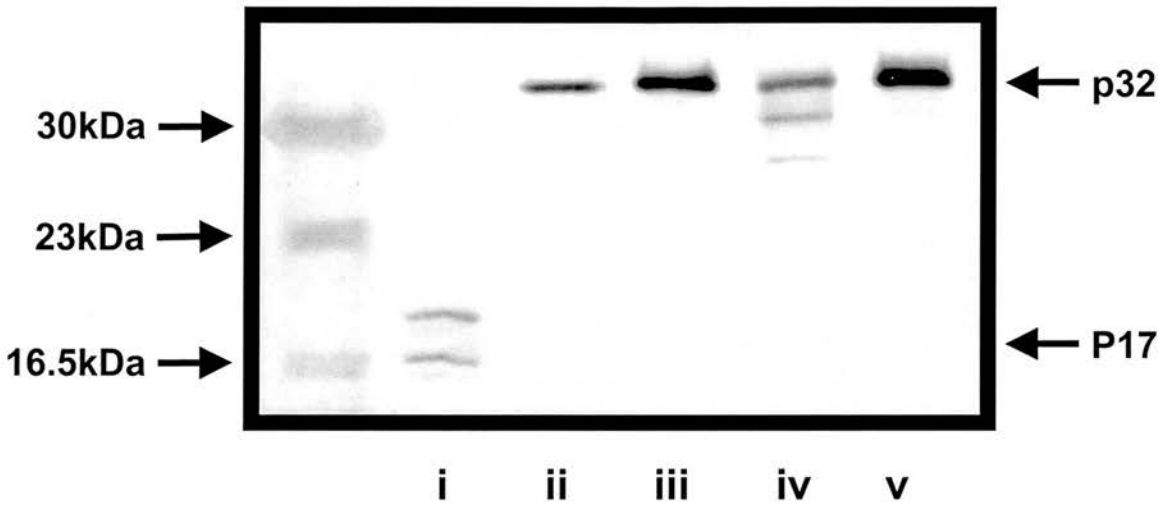


Figure 4.1: Caspase-3 is not activated as platelets age in culture. Cytosolic extracts taken from fresh platelets **(iii)**, platelets aged in the absence of serum **(iv)**, or platelets aged without serum but with calpeptin **(v)** were probed for caspase-3 by Western-blot with a pAb that recognised both the 32 kDa (p32) precursor and the 17 kDa (p17) subunit of the activated enzyme – note lack of the latter. As positive controls, cell lysates were prepared from untreated Jurkat T-cells **(ii)** which exhibited low levels (10%) of constitutive apoptosis as judged by Giemsa stained cytospins, and those induced to undergo apoptosis **(i)** following a 5 h treatment with staurosporine (2μM) (approx. 65% apoptosis). Positions for the 30, 23, and 16.5 kDa mass markers were determined with the use of Rainbow Markers.

4.1.4 Caspase-3 activity is not detected in aged platelets with a fluorogenic substrate

Modifying the small peptide caspase inhibitors to contain a quenched fluorophore instead of the inhibitory -fmk domain allows the analysis of caspase activity within cell lysates by fluorimetry. Caspase cleavage of the peptide on the carboxy side of the aspartic acid releases the fluorophore, which is then free to fluoresce. Use of the DEVD-amino-fluoromethyl-coumarin (DEVD-AFC) substrate provides the preferred cleavage consensus for caspase-3. Preparation of the cell lysates for analysis necessitates a modified lysis buffer devoid of cysteine protease inhibitors, thus allowing caspase enzymatic activity. Treating Jurkats with cycloheximide (20μg/ml) overnight to induce apoptosis provided active apoptotic cytosols for positive controls. Typically 40 % Jurkat apoptosis was witnessed, by Giemsa stained cytospins, from which lysates were prepared and frozen until needed. As can be seen in Figure 4.2, specific caspase activity directed against DEVD can be detected in the Jurkat lysates, revealed by the inhibition on addition of the caspase inhibitors DEVD-fmk and zVAD-fmk, which gave a synergistic reduction of activity. However the combination of both inhibitors failed to completely inhibit all aspartase activity, suggesting non-specific substrate cleavage still occurred. Using the non-specific protease mixture Pronase, fluorescence counts are similarly detected indicating cleavage of the caspase substrate, and thus suggests apparent caspase activity can be mimicked by non-specific protease mediated cleavage. Fresh platelet lysates contained only non-specific activity, evidenced by the lack of any significant inhibition on using the caspase inhibitors ($p=0.56$). In fact, taking into account the equal protein loading, the magnitude of this non-specific activity within fresh platelet lysates equates to the 'remaining' activity within Jurkat lysates on using both zDEVD-fmk and zVAD-fmk. Importantly though, aged platelet lysates displayed little activity at all. Given the loss of protein per platelet on aging by general cellular degradation, and hence the increased numbers of cells required to compensate and produce an equal protein concentration within a lysate, this activity certainly represents an over estimate. Combined, these results confirm that caspases do not have a major role in the constitutive cell death of platelets, and hence complement recent studies on apoptosis-like events associated with platelet activation (Vanags *et al* 1997; Wolf *et al* 1999).

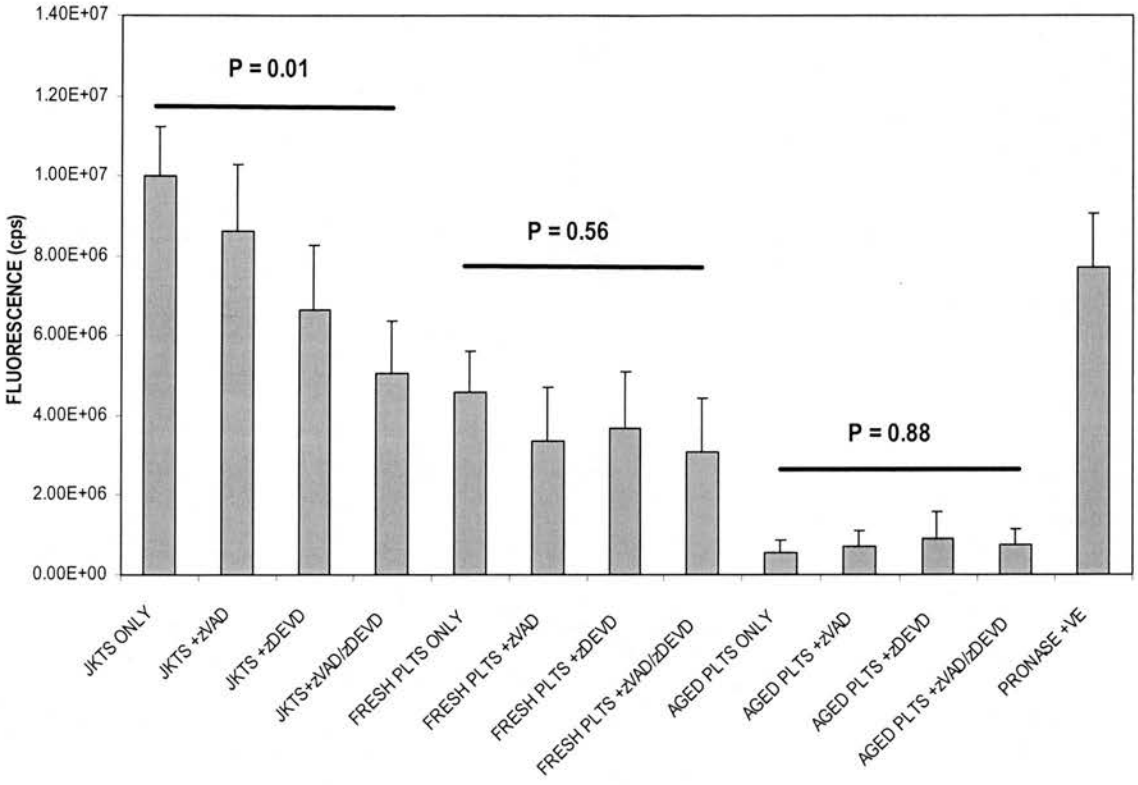


Figure 4.2: Fresh or aged platelet lysates do not contain active caspase-3. Fresh and aged platelets, or apoptotic Jurkats were lysed, incubated with caspase inhibitors as indicated, followed by incubation at 37°C with fluorescent caspase substrate. Reactions were stopped by placing all tubes on ice, before analysis of caspase activity in a fluorimeter using quartz cuvettes. Non-specific substrate cleavage activity was revealed by the lack of effect of caspase inhibitors. As a control for non-specific substrate cleavage, the protease mixture pronase was also tested. Data represent mean ± one S.D. of n = 3.

4.2 Aging platelets undergo biochemical changes indicative of an intrinsic death pathway

As platelets spontaneously undergo a constitutive death without any obvious external stimuli required, it could be argued that cell death was intrinsically controlled. Although the primary biochemical event initiating intrinsic cell death has yet to be fully elucidated, the downstream events have been extensively studied in many cell types. Given the absence of caspase-3 activation in aged platelets and the perceived relatively late ordering of caspase activation within a classical intrinsic death pathway hierarchy, we looked at events upstream with intent to find a possible block or divergence between platelet cell death and more classical forms. Intrinsic death normally proceeds through an increase in pro-apoptotic Bcl-2 family members, subsequently mediating mitochondrial dysfunction (Martinou and Green 2001). We have already demonstrated upregulation of pro-apoptotic Bax and Bak within aged platelets in the previous chapter, and hence investigated potential mitochondrial changes during platelet death.

4.2.1 Aged platelets progressively lose their inner mitochondrial membrane potential on aging

The opening of the permeability transition pore along with a loss of the inner mitochondrial membrane potential ($_{\Delta}\psi M$) has been proposed by some to precede and cause release of cytochrome-C (Zamzami *et al* 1996a; 1996b; Zamzami and Kroemer 2001), and by others to occur as a later event as a result of cytochrome-C release (Martinou and Green 2001, and references therein). This issue remains controversial and unresolved, but in either case cytochrome-C release leads to a subsequent amplification of caspase activation, and as such loss of $_{\Delta}\psi M$ still represents an early step in the intrinsic death pathway. Using JC-1, an electrochemical gradient-sensitive inner mitochondrial membrane-specific styryl dye, analysis of the level of mitochondrial transmembrane polarisation is possible by epi-fluorescent microscopy or FACS (Salvioli *et al* 1997). The JC-1 dye exists as two forms. In the presence of a mitochondrial potential it forms J-aggregates emitting principally orange fluorescence (~590nm). However, upon loss of the $_{\Delta}\psi M$, through either apoptosis or use of a

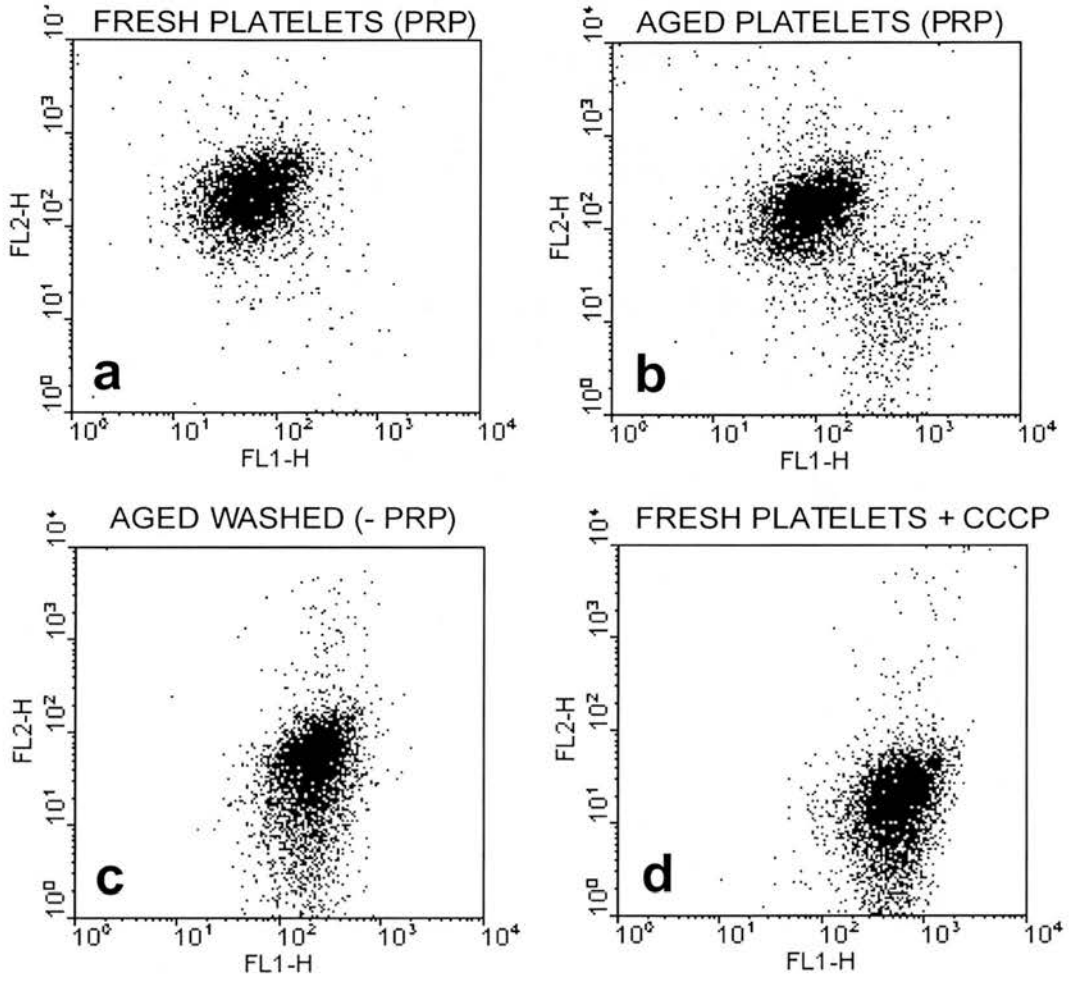
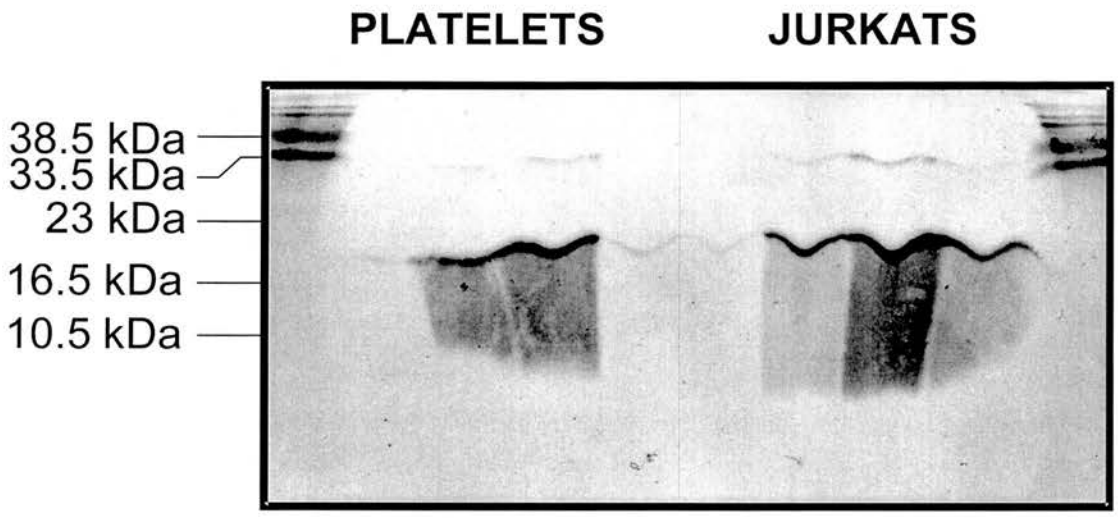


Figure 4.3: Senescent platelets show loss of inner-mitochondria membrane potential. Fresh PRP, aged PRP, or aged washed platelets were stained with the inner-mitochondrial membrane potential ($\Delta\psi M$) sensitive dye JC-1. **a,** Flow cytometric analysis of fresh platelets display a typical fluorescent profile of high orange fluorescence, indicative of an intact $\Delta\psi M$. **b,** In contrast, platelets aged in the presence of plasma show a small (~10%) distinct sub-population with a fluorescent profile indicative of a collapsed $\Delta\psi M$, i.e. loss of orange fluorescence with concomitant increase in green. **c,** Aged washed platelets show the entire population to have lost their $\Delta\psi M$. **d,** As a positive control, treatment with the protoionophore CCCP chemically collapses the electron gradient, whereby platelets emit similar fluorescent profiles to the aged cells.

respiratory chain uncoupler, such as the protonophore mCCCP, the dye disaggregates to monomers emitting a green fluorescence (~530nm). As can be seen a progressive loss occurs when platelets are aged either in the presence or absence of plasma, with cells obtaining a fluorescent characteristic identical to those deliberately uncoupled with mCCCP (Figure 4.3). This characteristic of platelet senescence appears to be the earliest change detectable within an aging population, preceding the CD62P and PS-exposure, and occurring before phagocytic clearance, a phenomenon very recently confirmed by Pereira *et al* (2002).

4.2.2 Release of Cytochrome-C in aging platelets occurs concordant with a loss of $\Delta\psi M$

As platelets were able to undergo a mitochondrial permeability transition comparable to conventional apoptosis, we investigated whether this was associated with a mitochondrial release of cytochrome-C. The conventional method of detecting release is to compare by Western-blot the levels of cytochrome-C contained within a mitochondrial pellet and a cytosolic supernatant following subcellular fractionation. This is usually accomplished by mechanical homogenisation, whereby selection of pestle size allows a defined clearance between wall and pestle enabling cells to be reliably "split open" without damage and leakage of mitochondrial contents. However, given the small size of platelets this method was unsuitable. By using a gentle hypotonic lysis fresh platelets could be readily separated into a cytosolic supernatant and a subcellular pellet, from which cytochrome-C could be immunoprecipitated. These samples showed minimal artefactual release of cytochrome-C from mitochondria to the cytosolic fraction due to preparation technique. Using this technique aged and freshly isolated platelets were compared with cycloheximide (20μg ml^{-1}) treated Jurkats, used as an apoptotic positive control. As can be seen, aged platelets contain mitochondrial-released cytochrome-C within their cytosol, in contrast to fresh platelets that contained cytochrome-C only within mitochondrial pellets (Figure 4.4). Equivalent results were seen in Jurkats, although cytochrome-C was still found within mitochondrial pellets of apoptotic cells, almost certainly due to a mixed apoptotic/non-apoptotic population.



Supernatant	+	+			+	+		
Pellet			+	+			+	+
Viable	+		+		+		+	
Non-Viable		+		+		+		+

Figure 4.4: Aged platelets release mitochondrial cytochrome-c into the cytoplasm. Fresh (viable) and aged washed (non-viable) platelets, as indicated, were lysed and cells fractionated by centrifugation to yield a cytosolic supernatant and a subcellular pellet. Both supernatant and solubilized pellet were pre-cleared with a control IgG before immunoprecipitating cytochrome-C. Cytochrome-C was detected by Western-blot analysis, with positive controls provided by treatment of Jurkats with cycloheximide. As can be seen cytochrome-C is detected within the pellet of viable cells, in contrast to the supernatant of non-viable cells.

4.3 Platelets undergo biochemical changes necessary for caspase activation, but do not activate caspases

As discussed in previous and present chapters, platelets undergo many changes indicative of cell death. In summary, aged platelets exhibit increased levels of proapoptotic Bak and Bax, undergo loss of $_{\Delta}\psi M$, release cytochrome-C, contain caspase-3, undergo plasma membrane changes associated with cell death, and are selectively cleared by phagocytes. However, the combined data also suggest that caspases do not play a role in the constitutive cell death of platelets. It has previously been reported that platelets contain significant levels of caspase-9 that is readily activated by the addition of exogenous cytochrome-C and dATP to platelet lysates (Wolf *et al* 1999). It was therefore surprising in our experiments that caspase-3 was not activated within the aged platelets, especially given release of mitochondrial cytochrome-C, which represents the key initiator for formation of an active apoptosome.

4.3.1 Addition of Cytochrome-C and dATP to platelet lysates in a cell free system does not result in activation of caspase-3

In order to investigate the disparity of why caspase-3 was not activated within the aged platelets, we attempted to activate caspase-9 in fresh platelet lysates by adding exogenous cytochrome-C and dATP. This should have reconstituted an active cell free apoptosome resulting in the processing of caspase-3 to its active p12/17 form (Liu *et al* 1996). However, pro-caspase-3 remained unprocessed in excess of 3 h, whether looking at untreated control samples or those induced by cytochrome-C/dATP addition (Figure 4.5). This was in stark contrast to caspase-3 activation in Jurkat lysates where exposure to cytochrome-C/dATP reveals a clear progressive processing of caspases-3 pro-form to the p12/17 active fragments (Figure 4.5). Several reports have shown that calpain activity is able to cleave and inactivate caspase-9, and hence block caspase-3 processing (Wolf *et al* 1999; Lankiewicz *et al* 2000; Chua *et al* 2000). However, given the lack of Ca^{2+}, the presence of 1mM EGTA, EDTA, and calpain inhibitors such as leupeptin within the lysis buffer, it appeared unlikely that the calcium dependent calpains were activated and responsible for the inactivation of

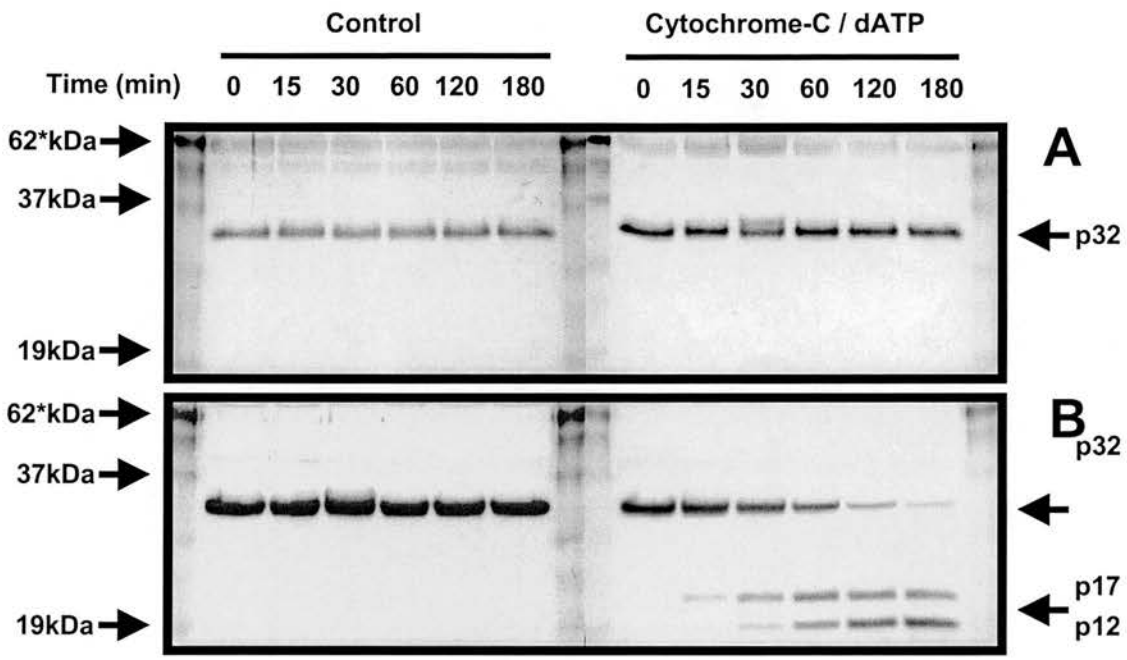


Figure 4.5: Caspase-3 is not processed to an active form within cytochrome-C/dATP induced cell free platelet lysates. Jurkat and fresh platelet cytosolic extracts were treated with buffer only (control), or cytochrome-C (10μg/ml) and dATP (1mM) and incubated at 37°C. At times indicated aliquots were removed and prepared for SDS-PAGE, with subsequent western blotting using a polyclonal anti-caspase-3 antibody. **A,** Platelet lysates containing equivalent quantities of pro-caspase-3 as Jurkats fail to process caspase-3 following cytochrome-c/dATP initiation. **B,** In contrast, identically prepared Jurkat lysates show progressive processing of the enzyme to the proteolytically active p17 and p12 fragments. The experiment was performed twice

caspase-9. In addition, the procaspase-3 bands showed no evidence of the 'tell-tale' 28kDa proteolytic "nibbling" indicative of calpain degradation, shown previously (Figure 4.1) and by others (Wolf *et al* 1999; Lankiewicz *et al* 2000; Chua *et al* 2000). However the data is still at odds with that of Wolf *et al* who showed cytochrome-C / dATP mediated the sequential activation of caspase-9 and caspase-3 within platelet lysates. Before investigating whether a potentially novel molecular inhibitor was responsible for blocking the formation of an active apoptosome in aged platelets, and given that the only other proteins known to be required for cell free caspase-3 processing are caspase-9 and APAF-1, we took a minimalistic view to see if any of these factors were missing from platelets.

4.4 Platelets contain APAF-1 but not Caspase-9

The previous report that platelets contain both key proteolytic components of apoptosome APAF-1 and caspase-9 used an in-house polyclonal antibody with Westerns developed by ECL (Wolf *et al* 1999). However, given the dubious intensity of the reported bands, absence of molecular markers, and after consultation with colleagues on the difficulty of blotting for, and low levels of caspase-9 within many cell types, we investigated the possibility that the data might have been generated from unchecked non-specific bands.

4.4.1 Freshly isolated platelets contain APAF-1 protein

Using Jurkats as a positive control, fresh blood platelets and megakaryocytes were analysed by Western-blot using a polyclonal anti-APAF-1 antibody. These blots confirmed that a single band around the apparent molecular weight of APAF-1 (130kDa) was clearly present within platelets, although Jurkats and MKs contained multiple bands close to this size that had merged into one on development, and many other non-specifics of different sizes (Figure 4.6). The blot had been blocked and washed using a 'standard protocol' of 2 % casein followed by 2 % casein / 0.1 % Tween-20, which has normally produced very low non-specific background binding. The previous published data had used 5 % marvel / 0.1 % Tween-20, which did not generally block as well as casein. However, as with all western blotting each antibody requires individual optimisation of all stages to

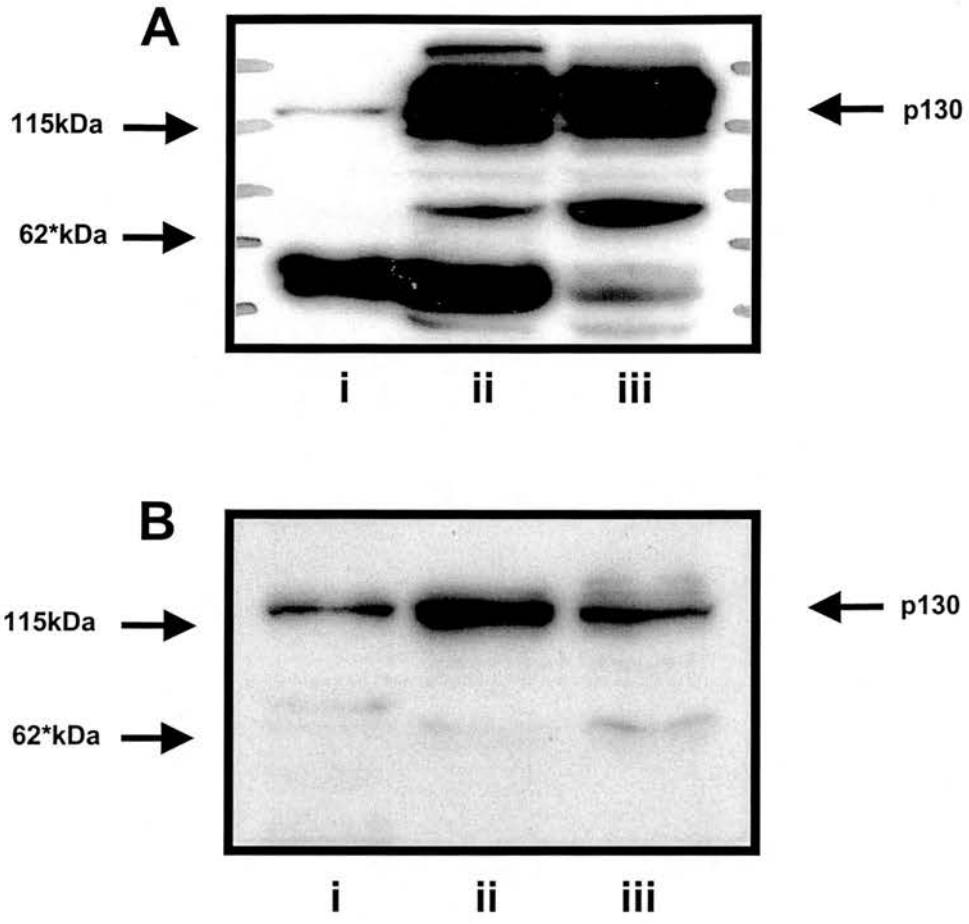


Figure 4.6: Platelets contain APAF-1. Whole cell lysates prepared from fresh platelets **(i)**, Jurkats **(ii)**, and MEG-01 MK cells **(iii)** were probed by Western blotting with a polyclonal anti-APAF antibody. **A,** Although platelets contained a band around the apparent molecular weight of APAF-1, 130kDa, many non-specifics were obtained within the control samples using 2% 0.1% Tween-20. **B,** Developing an identical blot under altered blocking and washing conditions, 5% Marvel 0.1% Tween-20, resulted in loss of non-specific bands, with only the 130kDa APAF-1 band present in all samples tested.

maximise the signal to noise ratio. Reanalysis of identical samples with conditions as before, except for the replacement of casein with marvel, produced a clean blot with only the p130 band present in all lanes (Figure 4.6). Strangely casein is the pure milk derived protein that actually is the blocking agent in marvel. Nonetheless, all samples contained ample quantities of APAF-1, although more was apparent within Jurkats. However, due to the very high actin content of platelets (>30 % of total protein) (Fox *et al* 1984) adjusting samples for loading by total protein content often leads to a 'skewed' lowering of the apparent amount of other proteins when comparing to other cell types.

4.4.2 Freshly isolated platelets do not contain caspase-9 protein

Using Jurkats as a positive control, caspase-9 was reliably observed by Western-blot using a commercially available polyclonal anti-caspase-9 antibody, and was found to migrate with an apparent molecular weight around 48kDa. However, we could detect no caspase-9 in fresh platelet samples, derived from three different donors on separate days, which was again in conflict with Wolf *et al* (1999) (Figure 4.7). Interestingly, the platelet sample in lane 4 contained a band just above the 38.5kDa marker, migrating at around 40-41kDa. Active caspase-9 is cleaved to an active fragment of 38kDa, but has been demonstrated to run with an apparent molecular weight of around 35kDa on discontinuous laemlli gels (Srinivasula *et al* 1998). Taking these sizes and given the clear reduction in protein from the non-specific band of ~85kDa in the same lane, the band almost certainly represents a proteolysed fragment, and is highly unlikely to be active caspase-9. Shown for comparison in the right hand panel are samples from the megakaryocytic cell lines MEG-01 and SET-2. Intriguingly both cell lines contained quantities of caspase-9 equivalent to Jurkats, thereby possibly implicating a loss or sequestration of the enzyme during platelet formation from the progenitors.

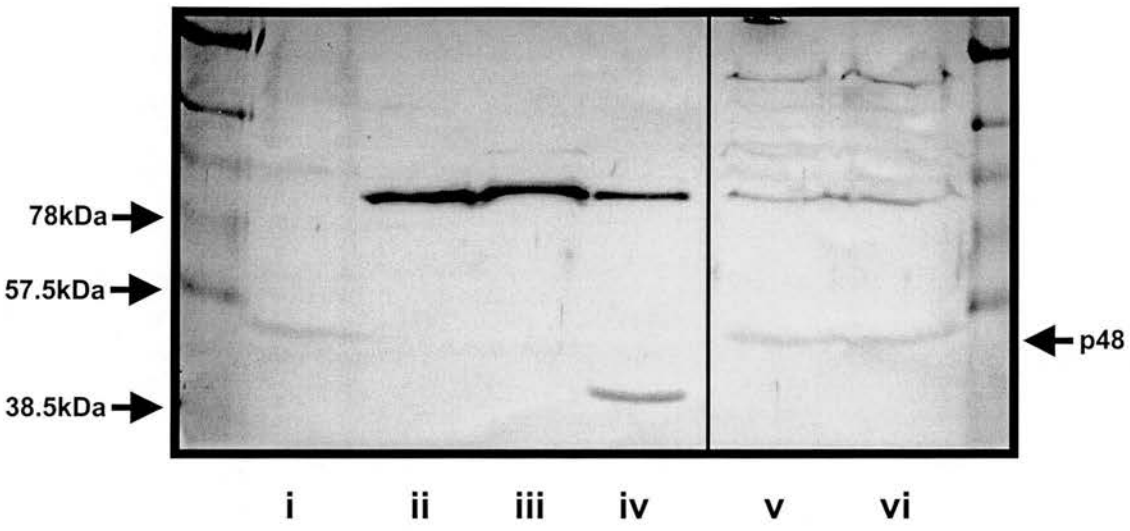


Figure 4.7: Fresh platelets do not contain caspase-9. Whole cell lysates prepared from Jurkats **(i)**, fresh platelets **(ii - iv)**, MEG-01 MK **(v)** and SET-2 MK cells **(vi)** were probed for caspase-9 by Western blot with a polyclonal antibody that recognised both pro-form and activated forms. One major band is detected around the apparent molecular weight of caspase-9, 48kDa, within the Jurkat and MK cell lines only.

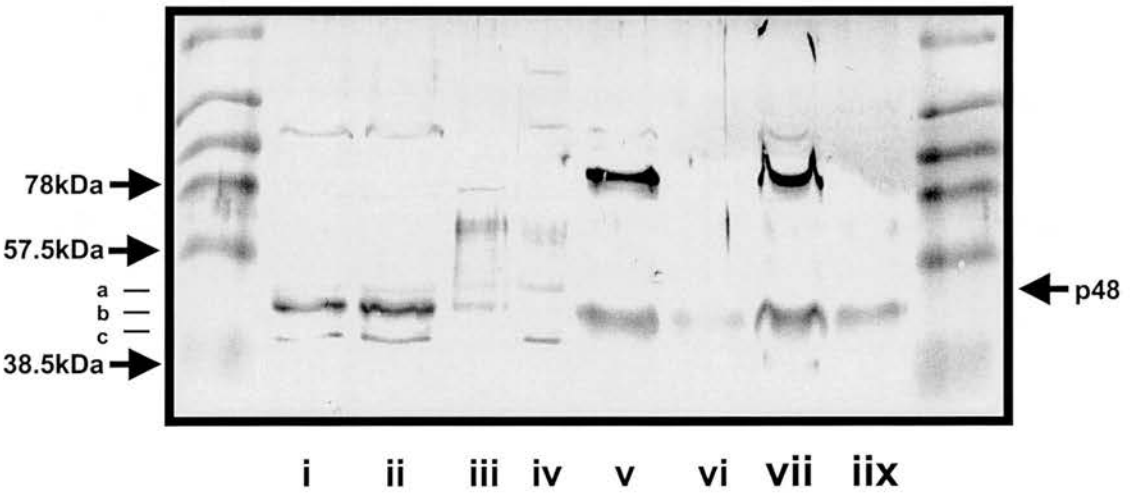


Figure 4.8: Lower stringency blotting reveals non-specific bands at a similar size to caspase-9. Whole cell lysates were again probed for caspase-9 using the polyclonal antibody, but with altered blocking and washing conditions. Membranes were blocked in 5 % Marvel and subsequently washed in 0.05 % Tween-20. Lanes contained Jurkats 1 **(i)**, Jurkats 2 **(ii)**, K-562 **(iii)**, HeLa's **(iv)** fresh platelets 1 **(v)**, aged platelets 1 **(vi)**, fresh platelets 2 **(vii)**, and aged platelets 2 **(iix)**. Again only the cell lines contained the p48 band (a). Interestingly, under these conditions a large diffuse band of ~ 45 kDa (b) is witnessed in all samples except the HeLa's.

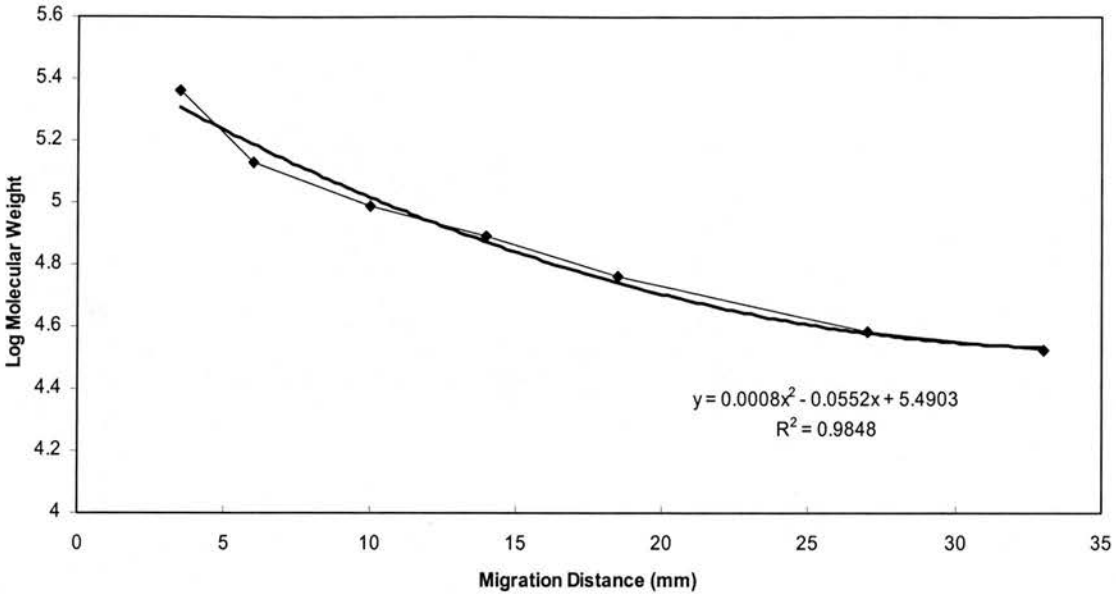


Figure 4.9: Analysis of the relative distance of migration to ascertain protein size. Rf values of protein standards ran on two 12% gels were measured and plotted. Using this plot, the apparent molecular weight of a band with a know migration distance, using a polynomial regression line of best fit, can be calculated. Hence, for the 'a' Jurkat band (Figure 4.8) migrating at an Rf = 21mm:

$$y = 0.0008(21)^2 - 0.0552(21) + 5.4903$$

$$y = 0.3528 - 1.1592 + 5.4903)$$

$$y = 0.3528 + 4.3311$$

$$y = 4.6839$$

$$\text{M.wt.} = 10^{4.6839} = 48295 \text{ Da}$$

Band	Migration distance (mm)	Apparent Molecular weight (kDa)
Jurkat upper	21.5	48300
Jurkat diffuse	23 - 24.5	45900 - 45000
Jurkat lower	26	41200
platelet diffuse	23 - 24.5	45900 - 45000
~78kDa platelet	12.5	88200

Table 4.4: Apparent molecular weights of bands detected by the polyclonal anti-caspase-9 antibody at 'low stringency'. All major bands detected with the polyclonal anti-caspase-9 antibody under low stringency conditions (Figure 4.8) were assessed for apparent molecular weight from their Rf values.

4.4.3 Western blotting under different conditions reveals three bands approximating the size of caspase-9

Western blots of the samples from above were probed under the altered blocking and washing conditions of 5 % marvel, followed by marvel / 0.05% Tween-20. Under these conditions three protein bands were found to migrate with a molecular weight comparable to that expected for caspase-9 (a,b,c), as can clearly be seen within the Jurkat positive control (Figure 4.8). However fresh platelets contained only one of these, the large diffuse central band (b) seen between the two finer bands (b,c) in the Jurkats. In addition, aged platelets also contained this central band (b). Speculatively, the diffuseness and intensity of this band seemed in line with the data published by Wolf *et al* (1999) for caspase-9 in platelets. To ascertain more accurately the apparent molecular size for each of these protein bands, relative distance of migration (Rf) was plotted against log of molecular weight (Figure 4.9). Calculating from two separate blots, it is seen that the large diffuse central band, appearing in platelets and Jurkats, consistently ran at a size of 45-46kDa, with the thin upper band at 48.3kDa and the lower thin band around 42kDa (Table 4.4). Given the close proximity of these three bands insufficient electrophoresis could fail to resolve the individual bands. In addition overexposure of a blot to photographic film by ECL could potentially "merge" the individual bands, or conversely a shorter exposure may only reveal the more intense diffuse central band. However, developing using the 4-chloro-naphol system allows visual monitoring as the blots develop, and hence allows a better control over the development end-point.

4.4.4 A specific monoclonal antibody confirms the identity and position of the caspase-9 band

In the meantime a more specific monoclonal antibody became commercially available, but manufacturers recommendations suggested it was only of use in cell lines specifically transfected to overexpress caspase-9. Nevertheless, given the disparity in the bands witnessed under the two conditions it was felt necessary to reinforce the data and confirm the upper 48.5kDa band to be the true caspase-9 band. Using the monoclonal antibody, western blotting of samples from the

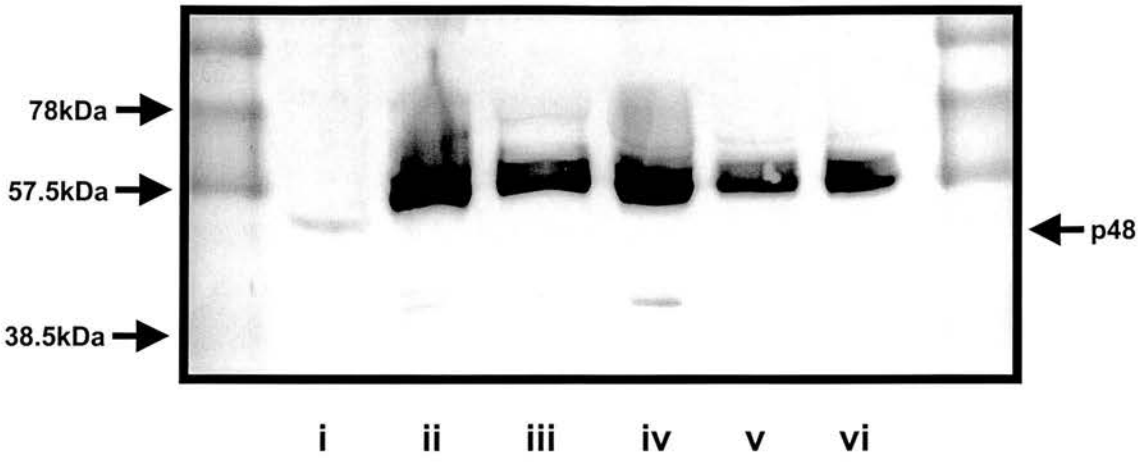


Figure 4.10: A specific monoclonal anti-caspase-9 antibody confirms position of the caspase-9 band. Jurkat **(i)**, and fresh platelet lysates **(ii-iv)** from Figure 4.7, excluding MK cell lines and with two additional platelet samples **(v-vi)**, were developed with an anti-caspase-9 monoclonal antibody recognising the pro-form only. Again only Jurkats contained caspase-9, which was found on calculating RF values to consistently run at 48.5kDa, and therefore in concordance with the 'clean' polyclonal blot. Platelets did however contain a large non-specific band of ~58kDa, but therefore clearly too large for caspase-9.

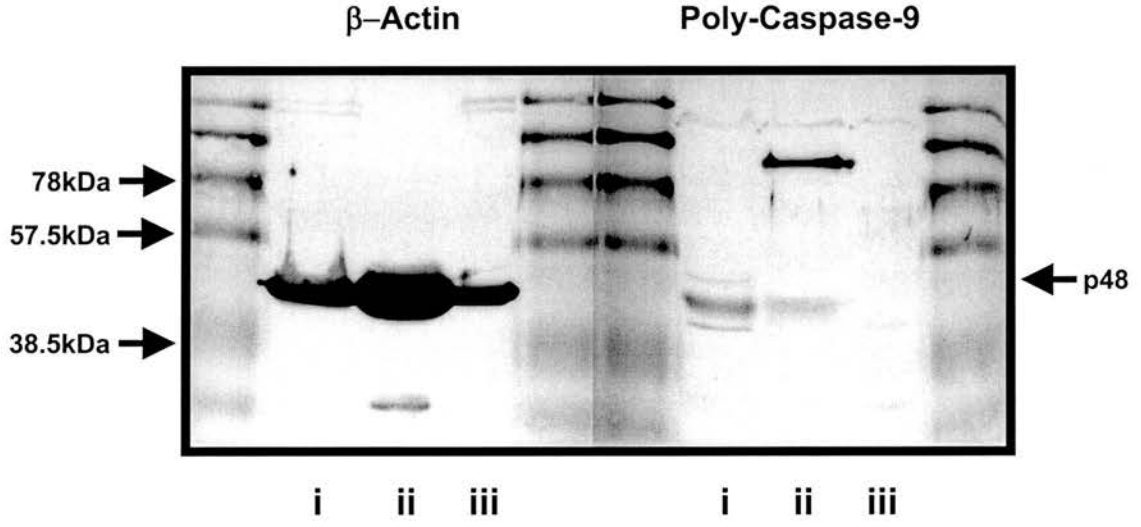


Figure 4.11: The diffuse band seen under low stringency conditions migrates with characteristics similar to actin. Jurkats **(i),** fresh platelets **(ii)**, and HeLa's were loaded in duplicate onto a single acrylamide gel and electrophoresed. Transferred proteins were stained separately for β-actin using a mAb, or caspase-9 using the pAb.

polyclonal blots, along with two other fresh platelet samples, again revealed no caspase-9 bands in platelet samples (Figure 4.10). In contrast Jurkats contained a band that, on plotting RF values, consistently ran at 48.6kDa (Table 4.5). The platelet samples did however contain a very broad non-specific band with an apparent molecular weight around ~55kDa, although this is clearly too large for caspase-9. Reassessment of the 'clean' polyclonal blot (Figure 4.7) by calculating RF-values gives the apparent molecular weight of the single band seen within Jurkat and MK cells to be in line with that seen using the monoclonal antibody, at an apparent molecular weight of 48.5kDa, and therefore in concordance of this band being the 'true' caspase-9 band, whilst the diffuse band occurring in platelet and Jurkats under lower stringency conditions appear to be non-specific.

Band	Migration distance (mm)	Apparent Molecular weight (kDa)
Jurkat singular	21	48600
~ 57kDa platelet	18 – 20	50900 - 57000
~ 38kDa platelet	25	40800

Table 4.5: Apparent molecular weights of bands detected by the monoclonal anti-caspase-9 antibody. All major bands detected with the monoclonal anti-caspase-9 antibody (Figure 4.9) were assessed for apparent molecular weight from their Rf values.

4.4.5 Western blotting for β-Actin in parallel with caspase-9

Interestingly the apparent molecular weight of the diffuse band and characteristic intensity and shape seen under the 'less stringent' conditions equates to that regularly seen by the group when blotting for β-actin. A single running gel was loaded in duplicate, electrophoresed, and after transferring to PVDF the membrane was blocked and carefully cut into two. In parallel, each was stained for either β-actin or caspase-9, and the two membranes were realigned (Figure 4.11). The large diffuse band occurring in Jurkat and fresh platelet samples on the membrane developed for caspase-9 ran with an apparent molecular weight, diffuseness, and shape akin to that seen on the membrane stained directly for β-actin. However, given the greater amount of β-actin detected within the platelets in comparison to the Jurkats, if the caspase-9 antibody was cross reacting to this, one would have expected the platelet band in the anti-caspase-9 developed blot to have been more intense.

Nevertheless, to confirm the band to be a non-specific cross-reactivity to β-actin we attempted to use immunoprecipitation to selectively clear samples. Unfortunately despite many attempts and adjustments to specific protease cocktails and more general lysis conditions, the logistics of IP resulted in loss of all bands. In fact simply slow manipulation of protein samples not kept on ice resulted in loss of immuno-detectable caspase-9. Assumedly, the epitopes recognised by the antibody are very sensitive to proteolytic degradation. Taken with the fact that platelets are known to contain large quantities of elastase, cathepsins, and calpains, and Jurkats to contain high levels of the serine protease granzyme-B, the proteolysis can only be grossly exaggerated. Speculatively, given the large amount of β-actin within cells, low amounts of caspase-9, and close apparent molecular weight of the two, the possibility arises that the published work could represent unchecked non-specific binding to β-actin.

4.4.6 Two-dimensional electrophoresis confirms non-specificity of the platelet band

Two-dimensional electrophoresis (2D-E) is a technique allowing proteins to be separated by two parameters. Firstly, proteins are prepared to allow separation by isoelectric focussing. Amphoteric substances, such as proteins or peptides, possess an inherent electrical charge that is either positive or negative, essentially the sum total of all charges contained within the side chains of its constituent amino acids. Loading of proteins onto a pH gradient with an applied electric field enables migration of proteins through the gradient towards either the cathode or the anode until they reach a position within the pH gradient where their net charge is zero. This pH value is known as the isoelectric point. Secondly, the separated proteins are equilibrated with SDS to allow the conventional SDS-PAGE "dimension" to be run, allowing separation by molecular weight. This final gel can then be transferred to membrane and probed with antibody, essentially allowing 2D-Immunoblotting (2D-IB).

To resolve the disparity between our observations for an absence of caspase-9 with the previously published data suggesting its presence we employed 2D-IB, developing with the polyclonal antibody. For Jurkat samples, Westerns were developed under the 'stringent' conditions, as in Figure 4.7, to positively identify caspase-9. Conversely platelet samples were developed under the 'less

stringent' conditions, as in Figure 4.8, to allow detection of the (non-specific) 45-46kDa band. Although not specifically published for platelets or available from the SwissProt database, the isoelectric point of caspase-9 can be calculated empirically to be 5.8. Platelet samples developed by 2D-IB contain three major resolved spots running at around 85, 70, and 45kDa, all of which correspond to the same sample run in only 1D SDS-PAGE on the right hand side (Figure 4.12; arrows). However, the pI of all three spots was around 7.5, and consequently inconsistent with caspase-9. In contrast, the Jurkat samples contained one major resolved band with an apparent molecular weight of 48.5kDa and a pI of around 5.7, both in accordance with it being caspase-9 (Figure 4.12). Although the data does not confirm the identity of the unknown platelet band, it does support the idea that the band reported by Wolf *et al* (1999) could have been a non-specific cross-reaction, highlighting fundamental weaknesses in 1D Western blotting, and an absolute need to accurately report apparent molecular size when part gels without markers are presented.

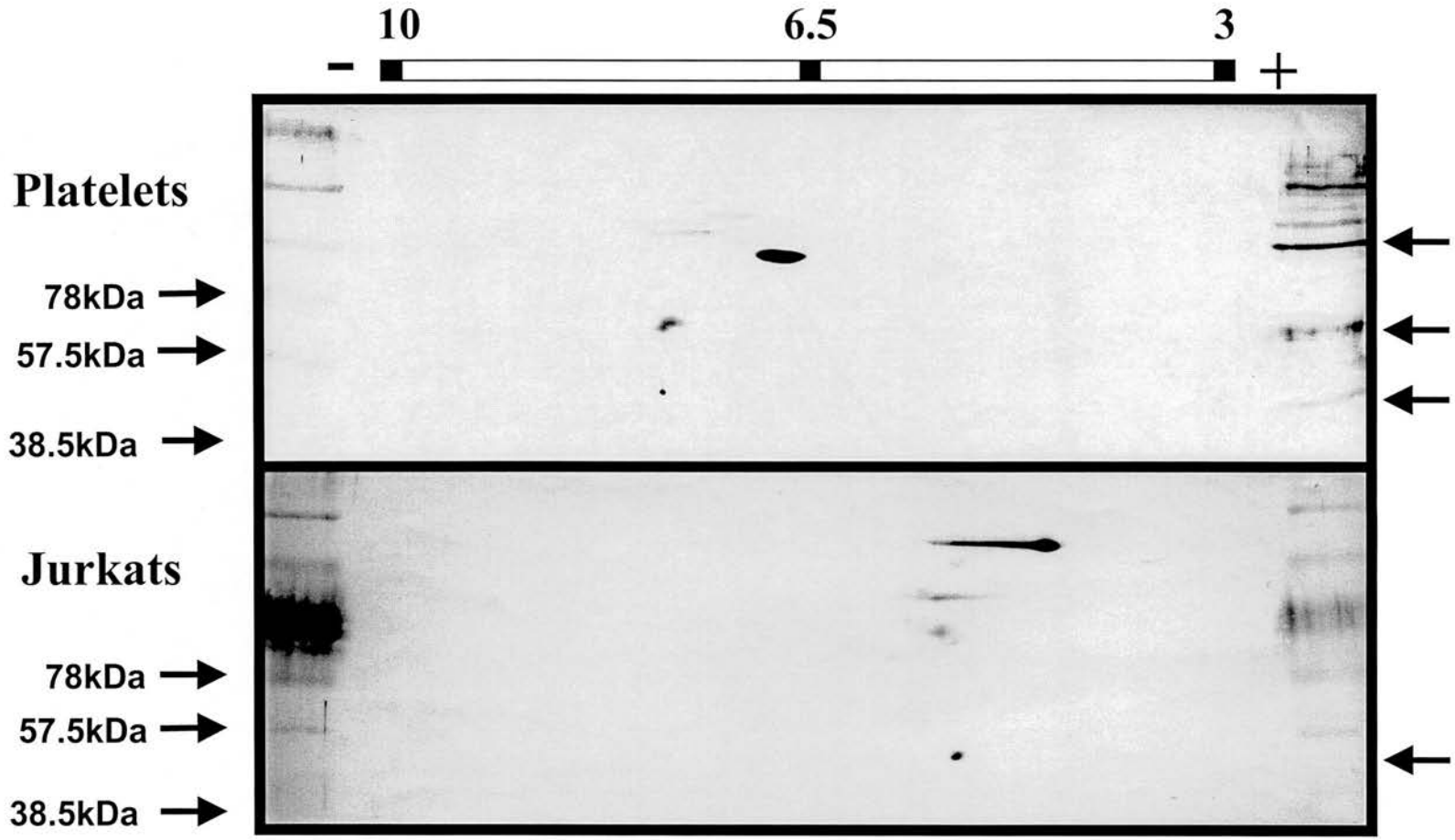


Figure 4.12: Two dimensional electrophoresis immuno-blotting detects a platelet protein at an incorrect pI for caspase-9. Fresh platelets and Jurkats were specifically prepared for 2D-E and subjected to first and second dimension separation followed by western blotting. Membranes were developed using the polyclonal caspase-9 antibody with the platelet membrane blocked and washed using 5 % Marvel / 0.05 % Tween-20, and the Jurkat blot blocked and washed with 2 % Casein / 0.1 % Tween-20. Three major spots are found in the platelet sample, aligning with non-specific bands detected in samples ran in one dimension only (arrows), but all at too high a pI for caspase-9 (5.8). However, the Jurkats sample contains one major spot at the apparent molecular weight (48.5kDa) and the calculated pI of caspase-9 (arrow).

4.5 Direct addition of human recombinant caspase-9 to platelet lysates reconstitutes an active apoptosome resulting in caspase-3 processing

Having identified caspase-3, cytochrome-C, and APAF-1 in cytosolic fractions of fresh platelets it remained for us to functionally confirm that the absence of caspase-9 was the sole reason platelets could not form an apoptosome, and hence activate caspase-3. Human recombinant caspase-9 (0.1 U/mL), cytochrome-C, and dATP were added to the same preparation of fresh platelet lysates as used in Figure 4.5. This single addition to the lysate resulted in the clear progressive processing of caspase-3, which must reflect a reconstitution and activation of the apoptosome (Figure 4.13). Although processing of the caspase-3 proform is not as complete as in the Jurkat cell free lysates presented previously, this almost certainly reflects the relatively low, and potentially limiting amount of caspase-9 added. Taken together this set of data definitively identifies caspase-9 as the single factor missing from platelets, preventing them from activating caspase-3, and hence committing them to a caspase-independent death program.

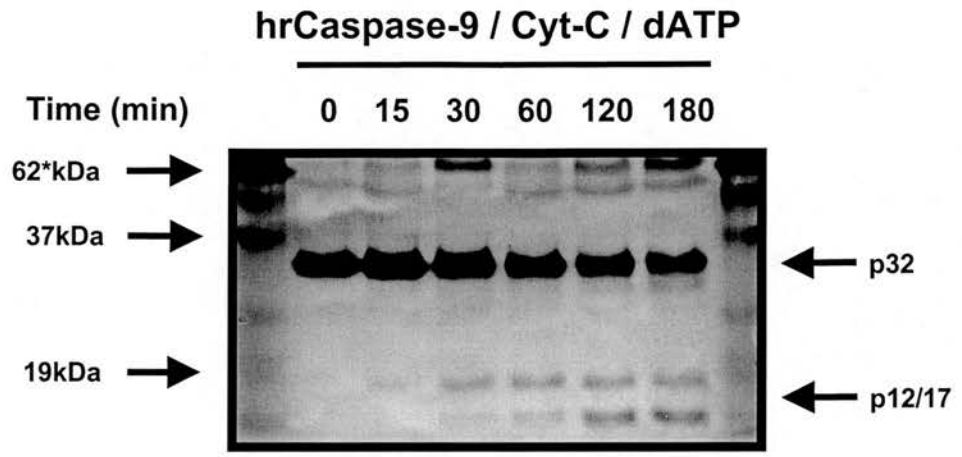


Figure 4.13: Direct addition of human recombinant caspase-9 reconstitutes the apoptosome. Human recombinant caspase-9 (0.1 U/ml) was added to fresh platelets lysates derived from the preparation as used in Figure 2, cytochrome-C and dATP added, the sample incubated at 37°C and aliquots removed at times indicated as previously described. In contrast to before, clear processing of caspase-3 to its active subunits can now be witnessed within the platelet lysates, indicating a reconstitution of the apoptosome.

Discussion

Despite an underlying paradigm against investigating caspase-independent cell death, many groups have successfully demonstrated cellular systems both *in vitro* and *in vivo* that can undergo a full death program, including clearance, without caspase involvement. This includes, in particular, the elimination in vivo of mature dendritic cells and B cells via MHC-II -dependent mechanisms (Drenou *et al* 1999), and T cells infected with HIV (Petit *et al* 2002). Further evidence is afforded from animal knockout experiments. Although caspase-3 deficient embryos show striking morphological perturbations of the forebrain, a study of post-mitotic neurons within the spinal cord and brain stem displayed caspase-independent death, with no effect on their ability to be recognized and cleared (Oppenheim *et al* 2001). Similar observations were seen with caspase-9 knockout mice, supporting the view that requirement for caspases might be dependent on tissue, cell type, age, and species (Oppenheim *et al* 2001). However, many of these systems have used synthetic caspase inhibitors or genetic ablation of caspases to reveal the pathways, and in this respect may reflect redundancy in cells ability to die, rather than a predetermined choice or requirement to be caspase-independent. Here we report that the intrinsic constitutive cell death program occurring in platelets on aging does not utilise caspases due to an absence of caspase-9, a key proteolytic component of the apoptosome.

This simple observation leads to some fascinating possibilities when combined with the fact that the platelets progenitor, the megakaryocytes (MKs), contain quantities of caspase-9 equivalent to Jurkats. Why do platelets not have caspase-9 when megakaryocytes do? One possibility is that caspase-9 is present in freshly formed platelets but is rapidly degraded by calpains, given that platelets are a rich source of these proteases and that they have previously been demonstrated to inactivate the precursor (Wolf *et al* 1999, Lankiewicz *et al* 2000, Chua *et al* 2000). Calpains have further been shown to be activated and function during a number of platelet activation events (Fox *et al* 1991), especially given their Ca^{2+} dependency and the well-characterised Ca^{2+} flux on platelet activation. Speculatively, given the multiple reversible activation events and interactions platelets undergo to maintain haemostasis within the circulation, repeated calpain activation may possibly result in the loss of caspase-9. However, two major reasons suggest this is not the case. Calpastatin is the

physiological antagonist of calpain activity within cells, preventing its activation due to increased Ca^{2+} alone, and requires cleavage to lose its inhibitory functions (Porn-Ares *et al* 1998). In addition, as shown by the caspase-3 blot (Figure 4.1) and work by others (Wolf *et al* 1999; Lankiewicz *et al* 2000; Chua *et al* 2000), calpain activity can be directed at caspase-3 resulting in a "tell tale" cleavage to protein bands of a size just less than the p32 proform. As evidenced from the same blot, freshly isolated platelets clearly do not show these bands indicative of calpain activation, and hence suggest that calpain mediated consumption of caspase-9 has not occurred.

Another possibility to explain the caspase-9 disparity between platelets and MKs is presented with understanding that platelet genesis is not a random fragmentation of the MKs cytoplasm, but a series of elegant active processes. As demonstrated by Italiano *et al* (1999), and based on much previous work, MKs actively extend long microtubule supported processes termed proplatelets (Becker and DeBruyn 1976). Open microtubule loops are thought to translocate through these processes, delivering platelet specific organelles such as α- and dense-granules to the proplatelet tips, where the closed microtubule coil forms to produce a mature platelet that is finally shed. The procedure appears to occur with precise control, given that platelets are rarely seen to contain endoplasmic reticulum or Golgi apparatus. In addition, further support for molecular compartmentalisation is provided by a recent report that has demonstrated platelets to contain a distinct subset of mRNAs derived from the MK (Lindemann *et al* 2001). From this understanding it could be proposed that MKs have the "ability" to specifically exclude caspase-9 from platelets during thrombopoiesis, albeit by an unknown mechanism, and for an unknown reason. This argument could of course be reversed, accounting for the fact that fresh platelets contain quantities of caspase-3 equivalent to Jurkats on a protein for protein basis, suggesting that the majority of the MKs caspase-3 protein is specifically delivered to the platelet. Separation of caspase-9 and -3 essentially blocks the intrinsic death program within the platelet, leaving only factors such as the mitochondrial released AIF or Smac/Diablo to act. If this separation was deliberate it makes sense for the cell to preferentially retain caspase-9, due to its lower copy number. Given that the cellular target of AIF has been shown to be the nucleus (Susin *et al* 1999), and Smac/Diablos' main target is the antagonism of

XIAP mediated caspase-9 inhibition (Du *et al* 2000; Verhagen *et al* 2000), these appear unlikely to be able to play a role in platelet cell death.

Therefore, if platelets possess most of the requisite machinery for caspase-dependent death, which is ubiquitous, what is the evolutionary pressure against platelets acquiring caspase-9 and -3 together? Given the association of caspase activation and PS exposure (Martin *et al* 1996; Vanags *et al* 1996), and the well-known procoagulant properties of PS, could it represent a safeguard against an accidental breakdown in haemostasis? This may seem odd given that in the previous chapter we clearly report PS exposure to occur during platelet death. However, senescent platelets are rapidly cleared, and as indicated in this chapter caspases do not appear to play a role in directing the surface changes leading to clearance of platelets. In addition, *in vivo* platelets may display "eat-me" signals and be phagocytosed before PS exposure occurs, resulting in their safe clearance. Could it be possible that under certain pathological conditions (blood sepsis?) blood borne toxins or factors could induce an intrinsic type death in platelets, especially as their anucleate nature limits their potential anti-apoptotic "counter response", which if caspase-9 was present would result in the mass procoagulant exposure of PS, certainly resulting in fatality. Similarly, given that procaspase-9 has a detectable albeit low activity (Thornberry and Lazebnik 1998), could this "smouldering" level ignite a full cascade, again given platelets limited ability to resynthesise, and hence maintain levels of caspase antagonists such as XIAP? If this were true caspase-independent death, or rather a blockade in caspase pathways, would be likely to exist in other anucleate cells, such as the erythrocyte. In fact caspase-3 activation has been demonstrated to be required, and is hence consumed during transition from the less mature nucleated polychromatic normoblast to the anucleate erythrocyte (Zermati *et al* 2001).

Perhaps it is this observation that leads to the third possibility for an absence of caspase-9 in platelets. Could platelet formation by the MK occur concurrent with, or require a form of altered or "hijacked" apoptotic program? Thus caspase-9 could be consumed during the generation of platelets. Examination of the caspase-dependent apoptotic blebbing that occurs in many cell types suggests that some parallels with platelet formation exist. This caspase-initiated process effects major biochemical

alterations to the cytoskeleton due to contractile forces generated by the actin-myosin system (Mills *et al* 1998), producing many individual membrane bound bodies into which the fragmented nuclear material has been actively localised (Coleman *et al* 2001; Sebbagh *et al* 2001). In addition, it has been reported that a major phenotype of the Bim k/o mouse, a pro-apoptotic member of the Bcl-2 family, is a profound thrombocytopenia (Bouillet *et al* 1999), whilst similarly the high level Bcl-2 expressing transgenic mouse shows a comparable phenotype (Ogilvy *et al* 1999). Given this evidence, albeit circumstantial, a preliminary investigation into a possible role for apoptosis in platelet formation from the MK was considered worthwhile.

Although confident the data supports an absence of caspase-9 in platelets, it has been previously reported by Wolf *et al* (1999) to be present. Our data demonstrate caspase-9 is not present both by Western-blot analysis, and by a functional reconstruction of the apoptosome. In addition, we have demonstrated that under altered blotting conditions a non-specific protein band around the molecular weight of caspase-9 can be detected in platelets, but which was shown by 2D electrophoresis to be of an incorrect pI. However, the Wolf paper displayed an ability to activate caspase-9 and subsequently caspase-3 in response to cytochrome-C and dATP. Another possibility to explain this disparity exists. The platelets used within the Wolf *et al* study (1999) were obtained from hospital platelet concentrates, usually destined for platelet transfusion. In the United States platelet concentrates are separated from whole blood by first preparing PRP, followed by collection of platelets after a second centrifugation, typically yielding around 5 x 10^{10} platelets per 50 ml bag (Vengelen-Tyler 1996). In Europe the buffy coat method is employed, involving centrifugation of whole blood to form a buffy coat, from which platelets are separated by an additional separation (Fijnher *et al* 1990; Murphy *et al* 1996). Although both methods produce platelet concentrate bags containing similar numbers of platelets, the difference in leukocyte contamination is large. The American PRP method yields around 10^8 leukocytes per bag, whilst the European buffy coat technique contains only 10^6 per bag, a hundred-fold variation. This equates to the American concentrates containing one leukocyte for every five hundred platelets. Given that it typically takes a hundred times the number of platelets to make a protein lysate of the same concentration as leukocytes, a sixth of the protein within the Wolf "platelet" lysate would have been derived from

leukocytes, known to contain caspase-9 (Webb *et al* 2000; Bantel *et al* 2001). We have always been aware of this and thus have ensured that platelets used for making lysates were subjected to multiple rounds of purification, as described under methods, resulting in around less than one leukocyte per one hundred and fifty thousand platelets.

Chapter 5 – Megakaryocyte Cell Death

Introduction

The terminal stage of MK maturation represents a unique and fundamental phenomenon of cell biology, yet relatively little is known about the process. Following multiple rounds of endomitosis to increase cell volume, and synthesis of vast amounts of platelet specific material, the MK breaks up into hundreds of mature blood platelets (Becker and DeBruyn 1976). However, this procedure is not a simple fragmentation of the MKs cytoplasmic content, but as elegantly shown by Italiano *et al* (1999) is an active process occurring with great precision, ensuring that the rich and unique cytoskeletal architecture of the platelet is properly formed. It is now almost universally accepted that platelet genesis occurs through intermediate morphological structures known as proplatelets. Proplatelets appear as long cytoplasmic extensions from the MK along which platelet sized nodes are observed, and contain multiple branch points to increase the number of tips. Thought to mainly utilise microtubule and actin components, platelet specific material is actively delivered to the proplatelet tips where the characteristic microtubule coil is formed and mature blood platelets are released (Italiano *et al* 1999). Although the basic "mechanics" of these events have been elucidated, any details of biochemical mechanisms driving such complex cytoskeletal rearrangements are highly speculative and based on the use of "blanket" cytoskeletal inhibitors such as taxol and cytochalasin D. In addition, it is important to realise that at the beginning of this study little was known about the physiological stimuli capable of driving proplatelet extension and platelet release. Progress in the MK field in general has been severely held back by the lack of a reproducible culture system that resulted in terminal maturation and platelet release. However, the relatively recent cloning and subsequent commercial availability of the lineage specific MK differentiation and maturation factor thrombopoietin (TPO) has kick-started a renewed interest in these fascinating cells.

Building on the suggestion within the previous chapter that caspase-9 may have been consumed by a "hijacked" apoptotic program during platelet formation, thus explaining its absence from platelets, we investigated whether inhibitors of the caspases could modulate platelet formation. Using a human megakaryocytic cell line and primary murine MKs, we found that platelet formation was dramatically inhibited by the poly-caspase inhibitor zVAD-fmk. Interestingly, the more caspase-

3 and -9 specific inhibitors zDEVD-fmk and zLEHD-fmk, respectively, failed to attenuate thrombopoiesis. Conversely, by treating MKs with Fas death receptor ligating agents, platelet production was significantly increased, presumably by induction of caspase activation. These findings were also reproduced in a novel *ex vivo* human bone core bioreactor culture system, producing platelets with morphology indistinguishable from blood platelets, and thus suggesting a strong physiological relevance for the observations. By using a cell permeable active caspase specific fluorescent dye we were able to identify active caspases within the main cell body of MKs bearing proplatelet extensions, but not the processes. Furthermore, the nuclear staining vital dye Hoechst 33342 revealed the caspase positive proplatelet bearing MKs to simultaneously display condensed nuclei typical of apoptosis. These observations were supported by TEM, which further revealed that MKs showing evidence of very early cytoskeletal and membrane rearrangements displayed nuclear condensation typical of an early stage of apoptosis, whilst those actively bearing proplatelets displayed a more marked condensation and fragmentation. In addition, direct enumeration of proplatelet bearing cells revealed the caspase inhibitor zVAD-fmk to inhibit formation of this phenotype, suggesting a requirement for caspases in driving, directly or indirectly, proplatelet extension.

Intriguingly, despite cells containing active caspases and displaying marked nuclear condensation and chromosomal fragmentation, proplatelet bearing MKs retain a mitochondrial transmembrane potential, and show evidence of active localisation of mitochondria to platelet sized nodes along the extensions. Furthermore, mature platelets shed from MKs under constitutive or Fas induced conditions did not expose PS at the cell surface, underwent typical agonist-induced platelet responses, and importantly were not phagocytosed by human MΦ. These data together strongly suggest that MK apoptosis not only occurs during platelet formation but is required, and may represent a novel form of "compartmentalised" apoptosis, given the disparity when compared to more typical apoptotic processes. This presents a fascinating opportunity to not only study two unique and phylogenetically ancient "co-opted" processes, but also to elucidate potential new therapeutic strategies for the control of thrombostasis.

5.1 Production of platelet-like particles strongly correlates with apoptosis

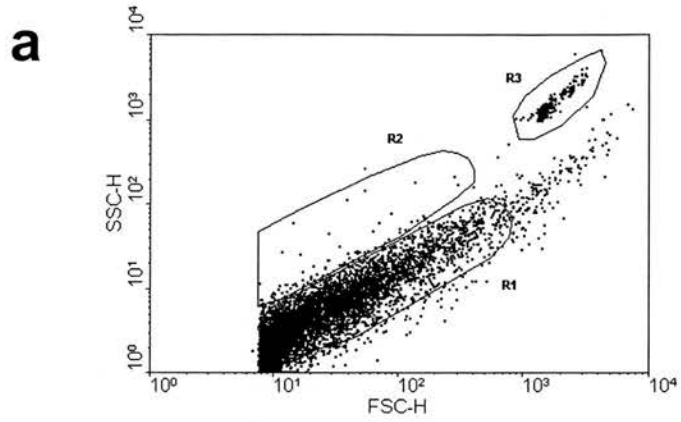
It has been previously suggested that a link exists between remnant denuded megakaryocytes displaying apoptotic morphology and the number of platelet-like particles (PLPs) found in culture supernatants (Zauli *et al* 1997). Although the apoptosis of the MK had been interpreted as being secondary to platelet formation, we investigated whether the apoptotic machinery was required, and whether the program of apoptosis itself was responsible for driving platelet formation.

5.1.1 Platelet-like particles can be enumerated by flow cytometry

The megakaryoblastic cell line MEG-01 has previously been shown to mature constitutively and spontaneously produce PLPs in culture (Ogura *et al* 1985; Takeuchi *et al* 1998). As the small size of platelets precludes the reliable use of manual counting methods, a flow cytometric technique was employed. This was felt to be superior to a Coulter counter, which infers size through the measurement of impedance changes across a defined opening, and hence can give no indication into the nature of the counted event. Using MK culture supernatant "spiked" with a known amount of 10μm polystyrene beads, to act as a fixed external control, the bead and PLP populations were easily divided by their FSC/SSC characteristics, allowing separate counts of each population to be made. From this ratio, given the fixed quantity of beads added, numbers of PLPs per ml were calculated (Figure 5.1). On comparing separate wells containing 2 $x10^5$ MKs each, it can be seen that following 18 h of culture the quantity of PLPs produced was reproducible, at around $1 \times 10^6 \pm 5 \times 10^4$ (Figure 5.1).

5.1.2 Pro- and anti-apoptotic reagents modulate production of PLPs

The megakaryoblastic cell line MEG-01 was treated for 18 h with a variety of pro-apoptotic agents, including staurosporine, etoposide, soluble Fas ligand, CH.11, lactacystin, ALLN, and anti-apoptotic agents including zVAD-fmk, zDEVD-fmk, zLEHD-fmk and calpeptin. Following treatment separated culture supernatants were enumerated for PLPs by flow cytometry, whilst remnant MK cells



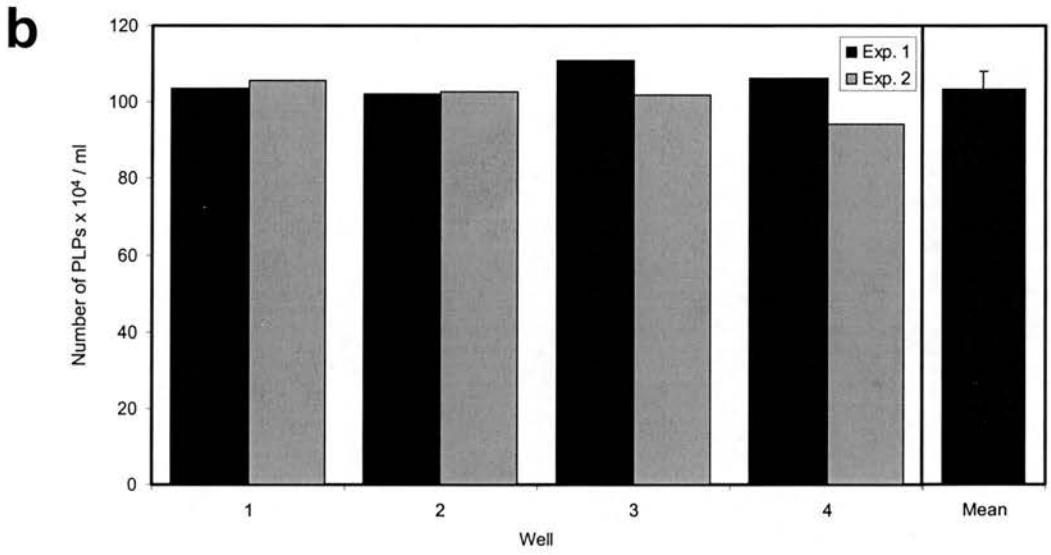


Figure 5.1: The MEG-01 MK cell line constitutively produces platelet-like particles (PLPs) at a constant rate. a, MEG-01 culture supernatants were enumerated for the number of PLPs produced by flow cytometry. By reference to a fixed number of beads, easily gated separately from the platelets (R3), absolute yields of PLPs falling within a typical blood platelet gate (R1) can be calculated. **b,** MEG-01 MKs at 4 x 10^5 / ml were plated into separate tissue culture wells. Following 16 h of culture the number of PLPs produced was assayed by flow cytometry. As can be seen, the rate of production remains relatively constant for a fixed number of MKs seeded. The right hand panel represents the mean from all wells. Data represent n = 2 from separate days, mean is ± one S.D.

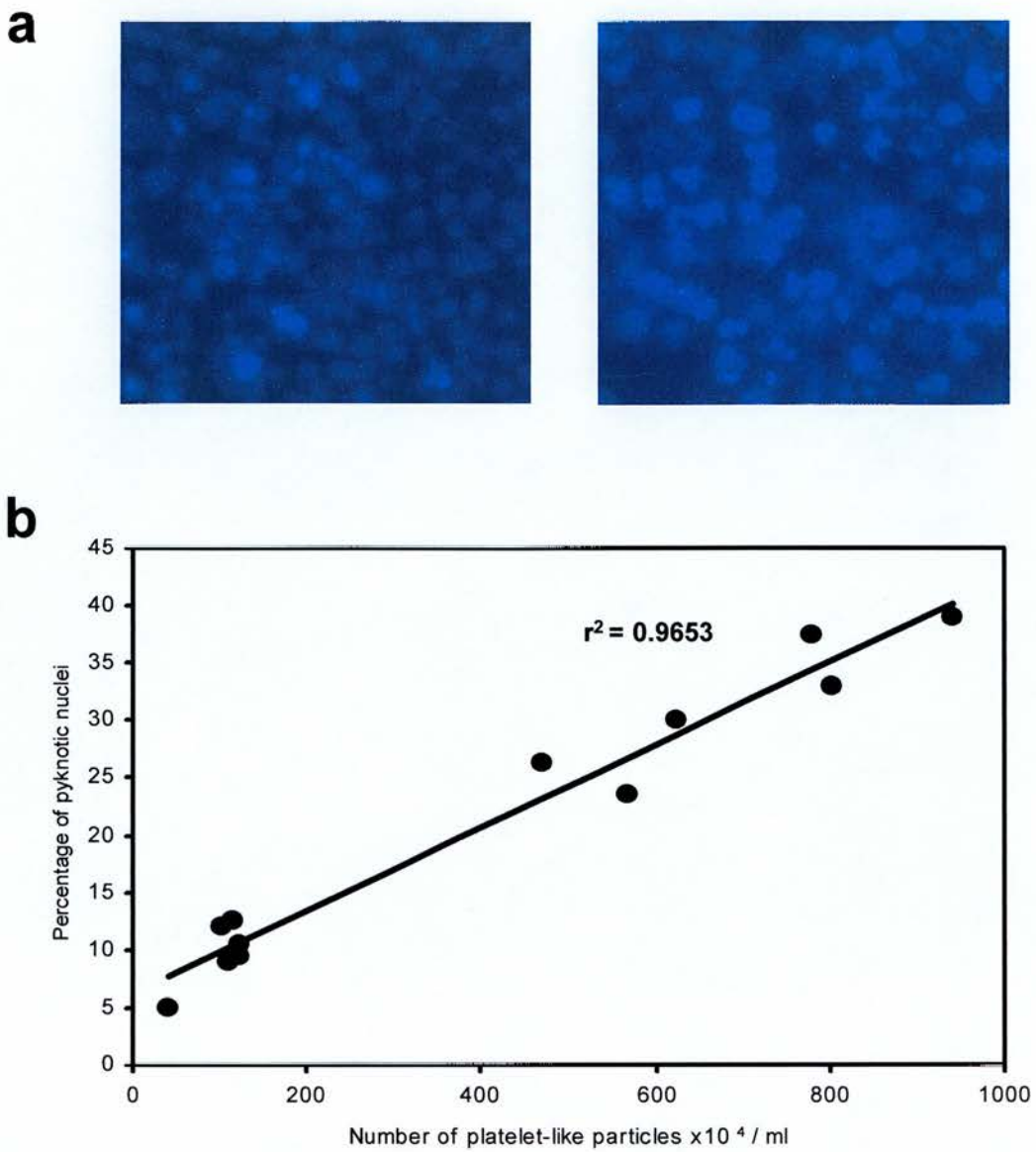


Figure 5.2: The number of platelet-like particles produced is strongly correlated to the level of MK apoptosis. MEG-01 MKs were treated for 16 h with a variety of pro- and anti-apoptotic reagents. **(a)** Remnant cells were assayed for the percentage of pyknotic nuclei using Hoechst 33342, and the number of PLPs produced enumerated by flow cytometry. **b,** A scatter graph of the level of apoptosis vs. the yield of PLPs revealed a tight correlation to exist, with an R^2 value of 0.97.

were counted for pyknotic nuclei, using the DNA binding vital dye Hoechst 33342. A tight correlation ($r^2 = 0.97$) was evidenced with a dramatic increase in the number of PLPs produced using pro-apoptotic agents and a consistent basal level of constitutive platelet production for controls (Figure 5.2). Likewise, a constant level of approximately 10% apoptosis was present within MKs maintained under control conditions. Interestingly, treatment with the poly-caspase inhibitor zVAD-fmk inhibited apoptosis and PLP formation to below this basal level. Given that untreated cultures spontaneously produced PLPs and spontaneously undergo apoptosis, and given the tight correlation between the two under "induced" conditions over a relatively short time course, the result suggested a close association between apoptosis and platelet production, and that a requirement for apoptosis in PLP production might exist. However, using the current assay it could be argued that any reagent which induced apoptosis would, depending on cell type, produce blebbed bodies, and given the absence of phagocytic clearance, cells will undergo secondary necrosis. Therefore, given the heterogeneous nature of platelet FSC/SSC characteristics, the possibility of necrotic cell debris and apoptotic bodies colocalising with any culture-derived platelets produced existed. Hence to further the study a more rigorous method of platelet enumeration was developed, as detailed in section 5.2.1.

5.1.3 Apoptosis is confirmed by DNA laddering

One of the key markers of apoptotic cell death is internucleosomal DNA cleavage, typically by the endonuclease CAD, and is commonly referred to as a "DNA ladder" because of the evenly spaced bands which appear when the extracted DNA is electrophoresed (Wyllie *et al* 1980). Interestingly, ladders were evident in DNA samples of untreated control MEG-01 MKs, whilst conditions that had previously increased apoptosis and PLP formation, such as treatment with the agonistic anti-Fas mAb CH.11, also increased the intensity of the ladder, given the equal loading conditions (Figure 5.3). Again the only treatment that reduced the intensity was the poly-caspases inhibitor zVAD-fmk, in which the ladder appeared fainter and most DNA remained retarded near the loading well, as indicative of un-cleaved genomic DNA. Intriguingly, the caspase-3 inhibitor zDEVD-fmk appeared to have no significant effect on the laddering occurring under control conditions.

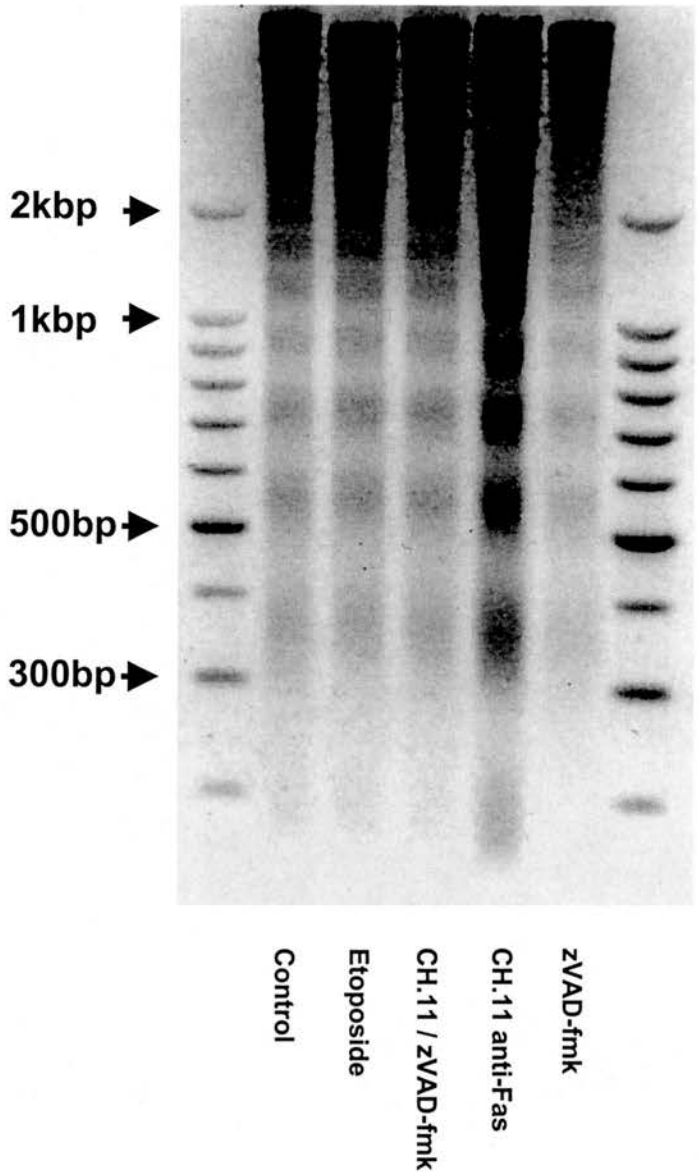


Figure 5.3: MEG-01 MKs undergo oligonucleosomal cleavage to produce a DNA ladder under constitutive conditions or following treatment with pro-apoptotic reagents. Purified mature MEG-01 MKs were cultured under control conditions, or with reagents as indicated for 12 h. Cells were collected and the genomic DNA extracted and electrophoresed on a 1.2% agarose gel, with bands visualised under UV light following ethidium bromide staining. All lanes contained the characteristic 200 bp ladder indicative of apoptosis, with increased oligonucleosomal cleavage witnessed following treatment with the agonistic anti-Fas mAb CH.11.

5.2 Culture derived platelets are functional and can be enumerated by several characteristics using flow cytometry

The positive identification of culture derived platelets is not a simple case of labelling for the lineage specific marker CD41/61, as material and debris derived from the parent MK inevitably contained the surface marker whether an actual platelet or not. Hence we developed an enumeration strategy based on typical functional responses to platelet-specific agonists such as thrombin or ADP.

5.2.1 Agonist-induced shape-change reliably indicates functional platelets

At "threshold" doses of agonist platelets still lose their normal discoid shape, becoming more spherical and extending membrane filopodia. Known as shape change the response is easily detected on a flow cytometer as an increase in SSC. Representing a reversible phase of activation at low agonist doses the platelets do not expose phosphatidylserine (Otterdal *et al* 2001). Therefore by using this response combined with propidium iodide and annexin-V staining it is possible to separate necrotic debris, apoptotic blebs, and dysfunctional PLPs from functional responsive platelets, thus allowing enumeration. Using freshly isolated blood platelets the cells can be seen to move from the "resting" gate R1 into the shape changed gate R2 on agonist stimulation (Figure 5.4). As expected, no binding of annexin-V or PI is witnessed, and as shape change represents a transient response only 80% of fresh platelets are shape changed at any given time. However, when MK culture supernatants are stimulated the shape-changed population (~60%) remains exclusively PI/AV negative, whilst the majority of the non-responsive population binds annexin-V (Figure 5.4). By "spiking" with 10μm beads (gate R3), to ascertain total number of particles (gate R1), and then undertaking agonist-induced shape change in the absence of beads (the relatively large size and amount of beads tended to interfere with the SSC signal from the platelets) it is possible to assay supernatants for total number of functional platelets per volume. Therefore throughout the chapter, reference to number of functional platelets is defined as PLPs capable of agonist-induced shape-change that remain PI/AV negative.

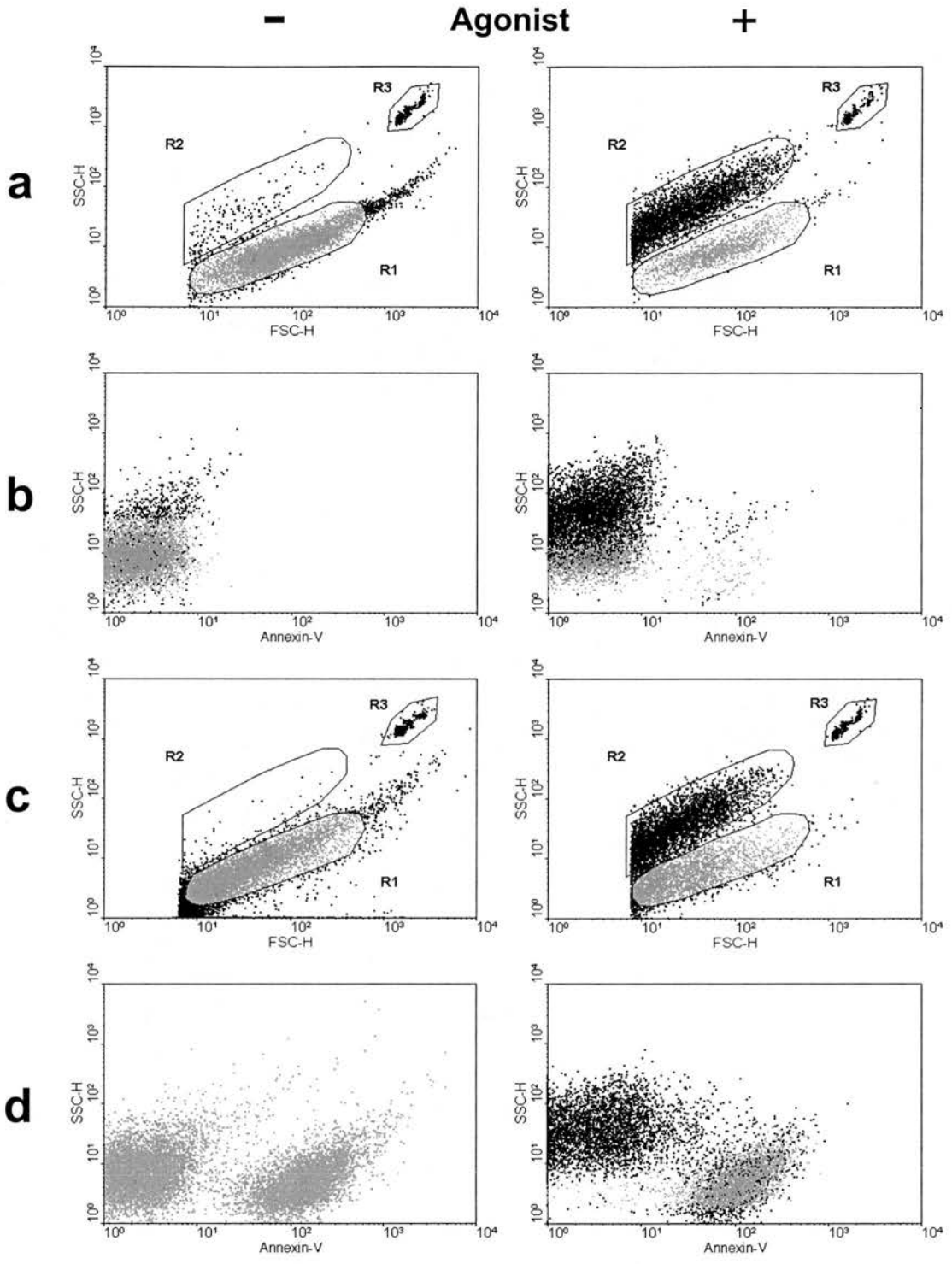


Figure 5.4: Functional platelets can be enumerated by a shape change response to platelet agonists. a, Freshly isolated blood platelets undergo shape change in response to agonist (as indicated), easily detected as an increase in side scatter (R1 > R2). Absolute counts of platelets were obtained by 'spiking' with a fixed number of 10 μM beads easily separated from platelets (R3). **b,** Freshly isolated blood platelets treated with or without agonist as indicated rarely bind annexin-V under the conditions employed. **c,** Culture derived platelets also undergo a shape change when stimulated, but in contrast contain an annexin-V positive population **(d)**, with only annexin-V negative cells undergoing thrombin induced shape change. Hence functional platelets were defined as those able to undergo agonist induced shape, but remaining annexin-V negative. All conditions contained PI as a dead cell gate, excluded from subsequent analysis.

5.2.2 Transient calcium fluxes are detected within the shape-changing population only

A well-characterised biochemical change occurring within platelets during the early reversible phases of activation, and particularly during shape-change, is the classic transient calcium flux (Robblee *et* al 1973). Using the visible light excitable Ca^{2+} sensitive dye Fluo-3, as opposed to the more commonly used UV excitable Fura-2, allows platelets to be analysed using a standard argon laser flow cytometer. Following stimulation of MK culture supernatants the shape-changed population only displays an increased Fluo-3 fluorescence within 30 s (Figure 5.5), indicative of a calcium flux. As reported in much platelet literature the majority of events return to basal levels within 90 s (Figure 5.5).

5.2.3 Culture derived functional platelets are not phagocytosed

To confirm the morphological and functional evidence that platelets produced by MEG-01 MKs undergoing apoptosis were viable, we investigated whether monocyte-derived macrophages (MDMs) would selectively clear non-functional platelets and MK fragments. Incubating MK culture supernatants with MDMs resulted in the selective clearance of all phosphatidylserine positive bodies, leaving a population of functional platelets able to demonstrate agonist-induced shape change (Figure 5.6). Interestingly, given the remnant MK cell body is known to be phagocytosed (Radley and Haller 1983), and the initial data that suggests inducing apoptosis increases production of PLPs, there appears to be a divergence from the more typical apoptotic program, which results in a difference of "edibility" between MKs and platelets.

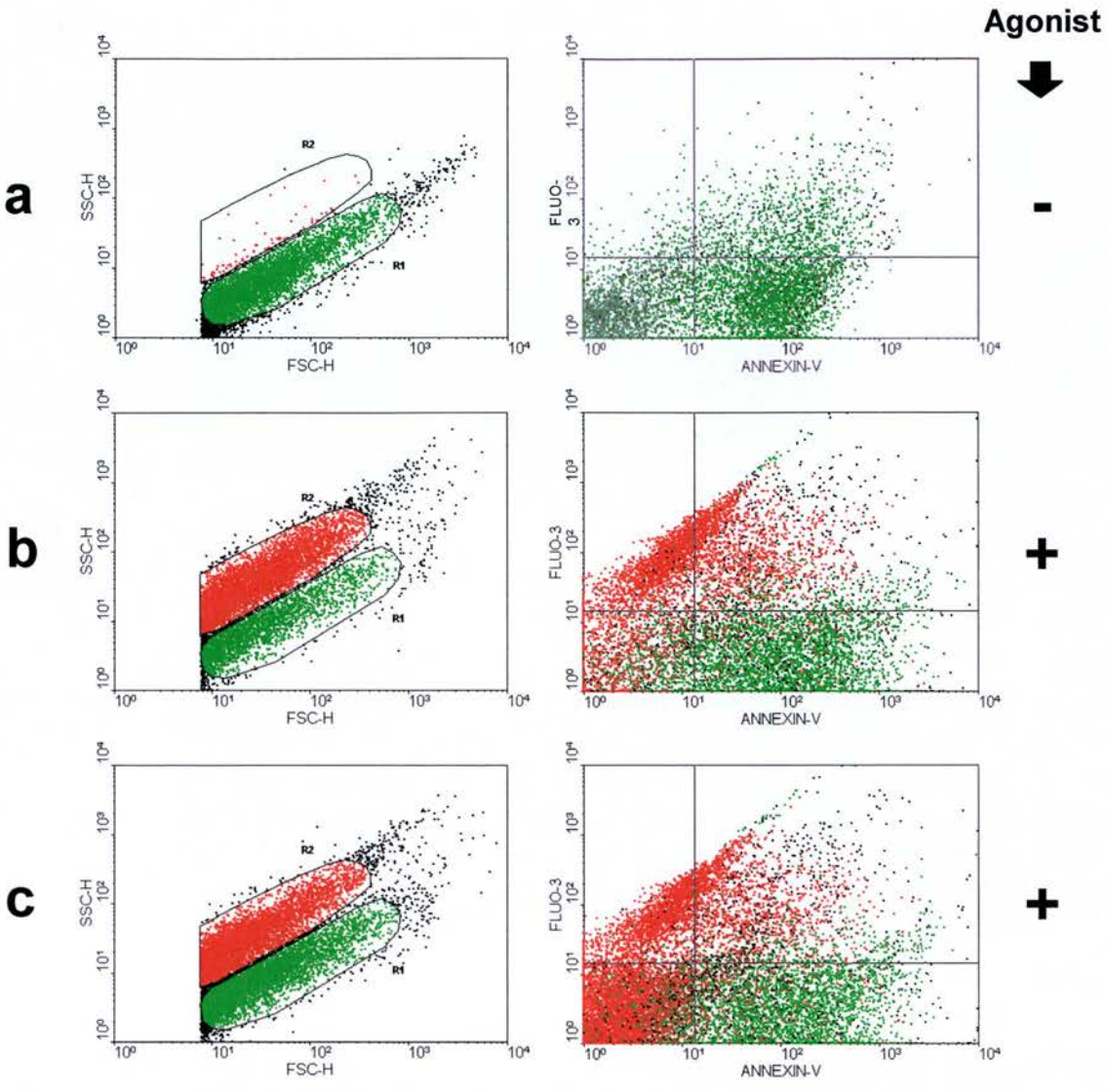


Figure 5.5: A transient calcium flux is detected within the shape changed population of MEG-01-derived platelets following agonist stimulation. MEG-01 MK culture-derived platelets were separated, stained with the Ca^{2+} sensitive dye FLUO-3, and analysed by flow cytometry. **a,** Without agonist stimulation platelets remained in a typical blood platelet gate (R1), whilst a subpopulation stained positive with annexin-V. **b,** On agonist stimulation with either thrombin or ADP, platelets shape changed as evidenced by an increase in SSC-H (events moving from R1 to R2). The majority of the shape changed population displayed an increase in FLUO-3 fluorescence indicative of an intracellular calcium flux. **c,** In line with the transient nature of Ca^{2+} fluxes, 90 s after stimulation most FLUO-3 positive events return to basal levels.

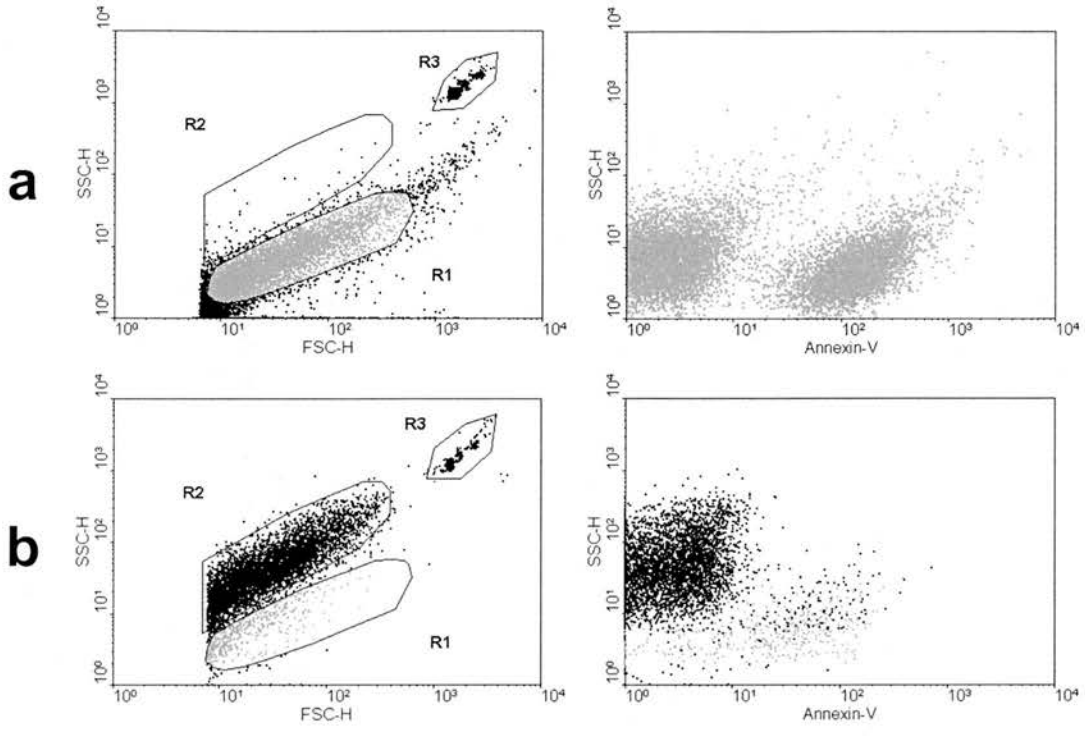


Figure 5.6: Platelets generated by MK are not phagocytosed by macrophages. Culture derived platelets from purified mature MEG-01 MKs were incubated alone **(a)**, or with human macrophages **(b)**, and subsequent culture supernatants analysed by flow cytometry. **b,** Incubation with macrophages resulted in the clearance of all annexin-V positive debris, with the majority of the remaining population able to shape change on agonist stimulation.

5.3 Production of functional platelets by human megakaryocytic cell lines is caspase-dependent.

In addition to the stringent requirement of all caspases for aspartic acid in the P_1 position, the P_4 position has been identified as the next most important determinant of the distinct substrate preference between various caspase family members (Thornberry *et al* 1997). Using a combinatorial approach Thornberry and colleagues developed a panel of individual caspase inhibitors with differential specificity between caspase groups. However, as with all peptide based enzyme inhibitors one should always hold concern over concentrations applied, with each inhibitor capable of inhibiting most family members at inappropriate concentrations. In general, a high level of cross-reactivity is particularly apparent within this family of inhibitors.

5.3.1 The broad spectrum inhibitor zVAD-fmk inhibits constitutive platelet production

By using the procedure for counting functional platelets detailed above, the effect of peptidyl caspase inhibitors on platelet production was investigated. Following 18 h of culture the MEG-01 cell line significantly produced around 50% less platelets following zVAD-fmk treatment (Figure 5.7). Interestingly, the caspase-3 inhibitor zDEVD-fmk, and caspase-9 inhibitor zLEHD-fmk had no effect (Figure 5.7). Given that MKs clearly display nuclear pyknosis and chromosomal fragmentation, as shown above, and that these two caspases represent the most common pathway by which CAD is activated (Liu *et al* 1997; Enari *et al* 1998), the result is intriguing. However, many studies have documented compensatory caspase activation enabling PARP cleavage and DNA fragmentation to occur, in particular by caspase-7 and -6 both able to function as efficient effectors (Kuida *et* al 1996; Zheng *et al* 2000). Culture of the MEG-01 cells with zVAD-fmk over longer time periods appeared to result in an apparent accumulation of the larger mature MKs, thus possibly implicating a zVAD-fmk sensitive step between final maturation and the initiation of proplatelet extension that is presumably caspase-9/3-independent.

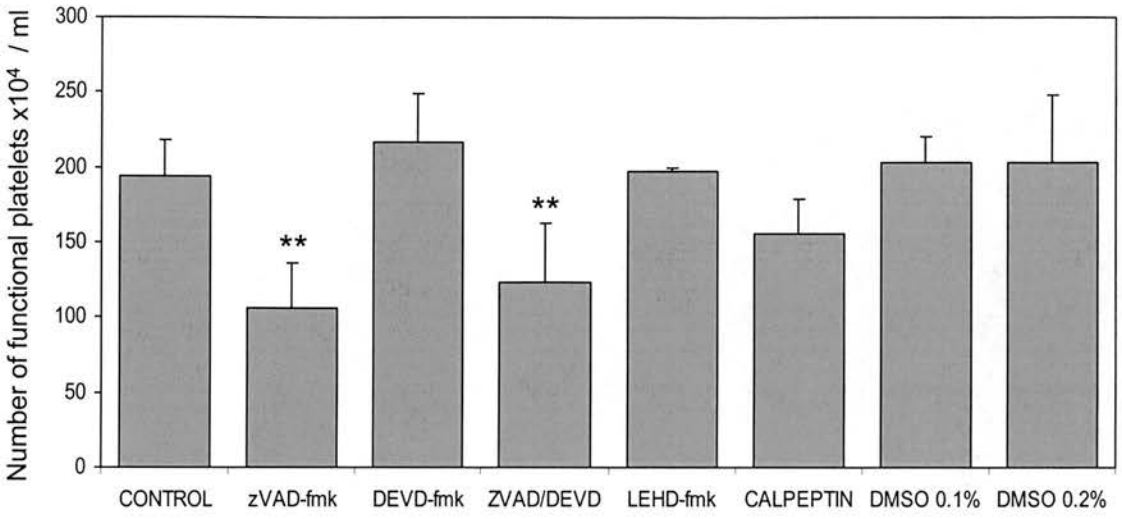


Figure 5.7: Production of functional platelets is significantly inhibited by the pan-caspase inhibitor zVAD-fmk. Purified mature MEG-01 MKs were cultured for 18 h with reagents or carrier DMSO alone as indicated, culture supernatants separated and assayed by flow cytometry for the yield of functional platelets as detailed in Figure 5.3. As can be seen the pan-caspase inhibitor zVAD-fmk significantly inhibited platelet production. However, the caspase-3/9 inhibitors DEVD-fmk and LEHD-fmk, respectively, had no effect and failed to synergise with zVAD-fmk. Data represent mean ± one S.D. of n = 4. ** represents $p < 0.02$ compared to control.

5.3.2 Inhibition of platelet production by zVAD-fmk is not through inhibition of cytokine processing, or miscounting due to prevention of shape-change

The first identified member of the caspase family was the interleukin converting enzyme-1 (ICE-1) (Thornberry *et al* 1992). Representing the archetypal example of a group-I caspase, the enzyme is highly sensitive to zVAD-fmk (Garcia-Calvo *et al* 1998) and is responsible for processing pro-IL-1 to its active form. To exclude the possibility that platelet production was IL-1-dependent, and that zVAD-fmk was mediating inhibition through prevention of pro-interleukin-1 processing, exogenous IL-1β (25 ng ml^{-1}) was added to cultures with and without zVAD-fmk. As can be seen, IL-1β failed to promote platelet production above control levels, and likewise failed to reverse zVAD-fmk mediated inhibition (Figure 5.8). In addition, as a previous study had suggested a requirement for caspases for a subset of platelet activation responses (Shcherbina and Remold-O'Donnell 1999), the possibility existed that zVAD-fmk treated cultures were producing platelets unable to shape change and therefore were not being counted. Pre-incubation of parallel fresh blood platelet samples with zVAD-fmk produced no affect in shape-change response in comparison to control platelets, by flow cytometry (Figure 5.9).

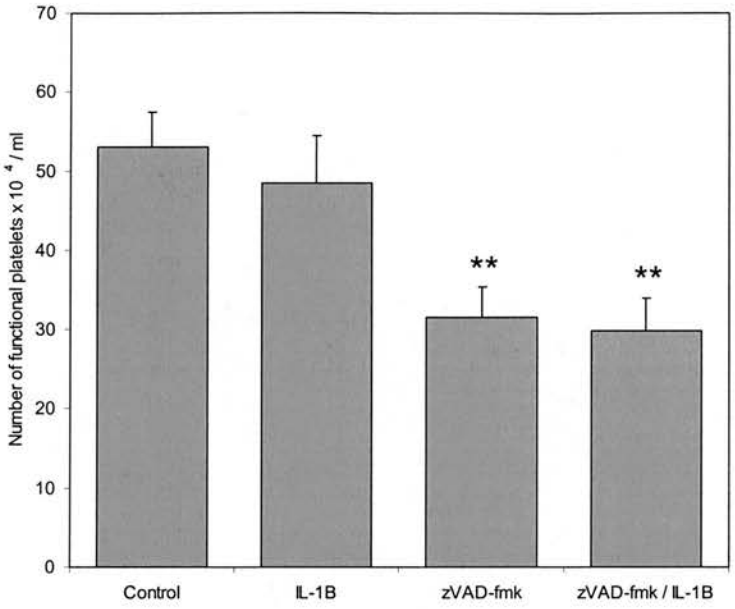


Figure 5.8: zVAD-fmk inhibition of platelet formation is not mediated through inhibition of cytokine processing by ICE-1. Purified mature MEG-01 MKs were cultured for 18 h with reagents as indicated, culture supernatants separated, and functional platelets enumerated by flow cytometry. Given that IL-1β failed to reverse zVAD-fmk inhibition or promote platelet formation, it suggests its action is not mediated through inhibition of IL-1β processing. Data represent the mean ± one S.D. of n = 3. ** represent $p < 0.02$ compared to control.

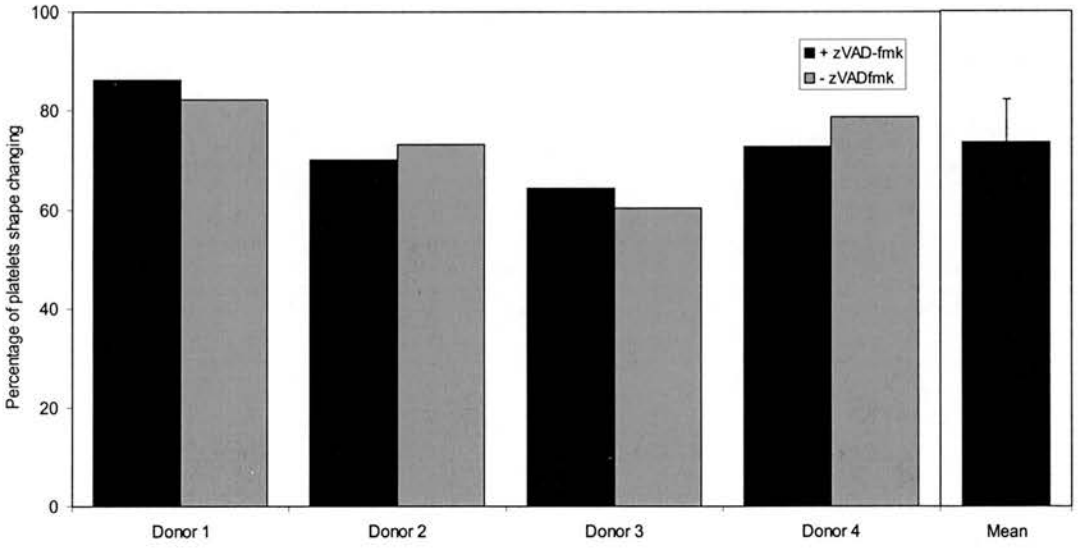


Figure 5.9: zVAD-fmk does not inhibit agonist-induced shape change. Fresh blood-derived platelets were pre-incubated with or without zVAD-fmk as indicated for 20 mins, before analysis of agonist-induced shape change by flow cytometry. The level of shape change was not significantly altered by zVAD-fmk treatment, and remained highly reproducible within each donor.

5.4 Production of functional platelets is induced by particular pro-apoptotic agents

As platelet production appeared to be caspase dependent, shown by the inhibitory effects of zVAD-fmk, we explored the possibility that deliberately driving a caspase-dependent cell death pathway could conversely promote platelet formation. However, staurosporine, which we had previously shown to induce apoptosis, is a poly-kinase inhibitor and thus is known to inhibit PKCα, recently shown to be required for proplatelet extension (Rojnuckarin and Kaushansky 2001). Therefore, and given that the Fas death receptor is an important regulator of early haematopoiesis (Santiago-Schwarz *et al* 1997; De Maria *et al* 1999; Josefsen *et al* 1999; Bryder *et al* 2001), we sought to determine if Fas ligand, a recognised initiator of apoptosis, could promote thrombopoiesis.

5.4.1 MEG-01 MKs express Fas on their cell surface as assayed by flow cytometry and epi-fluorescent microscopy

As a generalisation, most cells of myeloid origin have been demonstrated to express Fas on their cell surface. However, current literature contains little data about the presence of Fas or Fas ligand on MKs. Using the anti-Fas IgM antibody clone CH.11, MKs were labelled on ice, washed, and stained with an Alexafluor labelled secondary antibody. Using epi-fluorescent microscopy a subset of cells was seen to have specifically stained with CH.11. As can be seen a punctate pattern of fluorescence is witnessed, with some areas containing large patches of staining indicative of receptor clustering, and thought to be due to association with lipid rafts, as recently reported (Figure 5.10)(Gajate and Mollinedo 2001). In addition the same samples were assessed by flow cytometry for a more objective analysis of the number of cells expressing Fas and the relative level of surface expression. Typically, 42 ± 5% of the population expressed Fas, but a large variation in the level of expression existed (Figure 5.10).

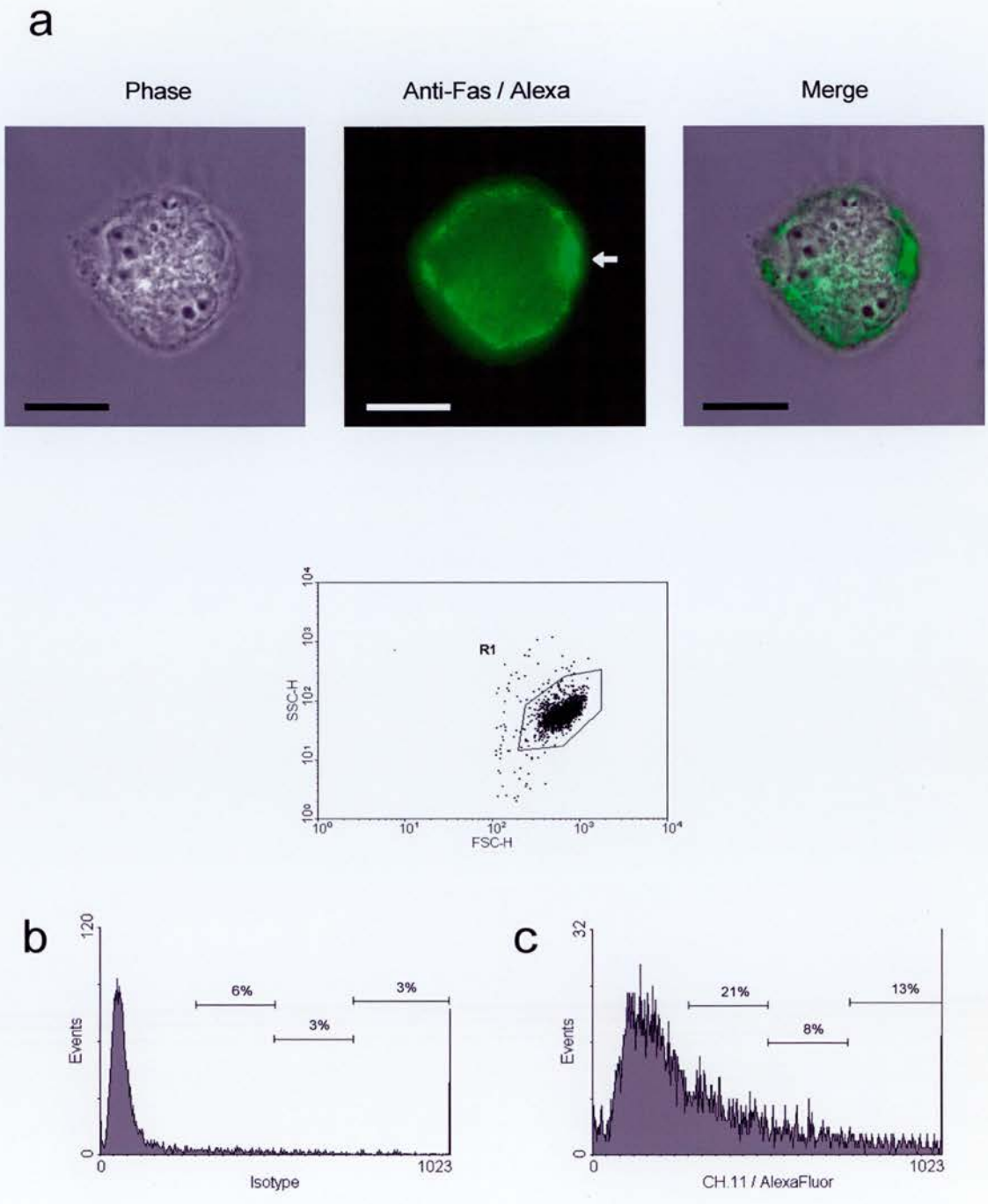


Figure 5.10: MEG-01 MKs express Fas on their cell surface. a, MEG-01 MKs where labelled on ice with the anti-Fas antibody CH.11 and stained with an AlexaFluor secondary antibody. Analysis by epifluorescent microscopy revealed Fas to be expressed on the cell surface (green). Some evidence of areas of receptor clustering is witnessed (arrow). Scale bar represents 20 μm. Cells stained with isotype control and secondary Ab (b), or CH.11 and secondary were also analysed by flow cytometry, revealing around 40% to stain positive for Fas, but with a large variation in the level of expression.

5.4.2 Platelet production is significantly increased by soluble Fas ligand, in a caspase-dependent manner

Given the presence of Fas on the surface of MEG-01 MKs we investigated whether the physiological agonist, in the form of human recombinant Fas ligand, could induce platelet production and apoptosis. Using a commercially available Fas ligand and enhancer system, speculatively thought to optimise receptor clustering using a FLAG- or histidine-tagged Fas ligand and an anti-FLAG/his antibody, we found platelet production was significantly increased (Figure 5.11). Interestingly, and in support of the inhibition of constitutive platelet production, this increase was revealed to be caspase-dependent by the inhibitory effect of zVAD-fmk (Figure 5.11). Furthermore, the ability to promote platelet production was somewhat restricted to Fas in that ligation of the TNF death receptor with TNF-α failed to promote platelet production (Figure 5.11).

5.4.3 The Fas agonistic antibody CH.11 effectively induces specific Fas ligation and platelet production

The archetypal IgM agonistic anti-Fas antibody clone CH.11 has been extensively studied and is well known to cause Fas receptor clustering and induction of cell death. MEG-01 cells were treated with CH.11 for 18 h and the functional platelets produced quantified. Platelet production was significantly increased around eight-fold above control levels at the relatively low CH.11 concentration of 50 ng ml^{-1} (Figure 5.12). Using the anti-Fas antagonistic antibody ZB4 we were reassuringly able to block the agonistic effect of CH.11, preventing increased platelet production, and hence indicating the action of CH.11 to be specifically via Fas ligation. Given the high cost of soluble Fas ligand and the amount required, and taking the data indicating the action of CH.11 to be specific, future studies conducted used CH.11 only. In concurrence with its effect on constitutive platelet production, zVAD-fmk was able to entirely reverse the induction. In contrast, zDEVD-fmk again was unable to reverse increased production. The small reduction evidenced using zDEVD-fmk was almost certainly cross reactivity to other caspase family members, as mentioned previously. If deliberately driving a known caspase-dependent death pathway results in increased platelet production, and if

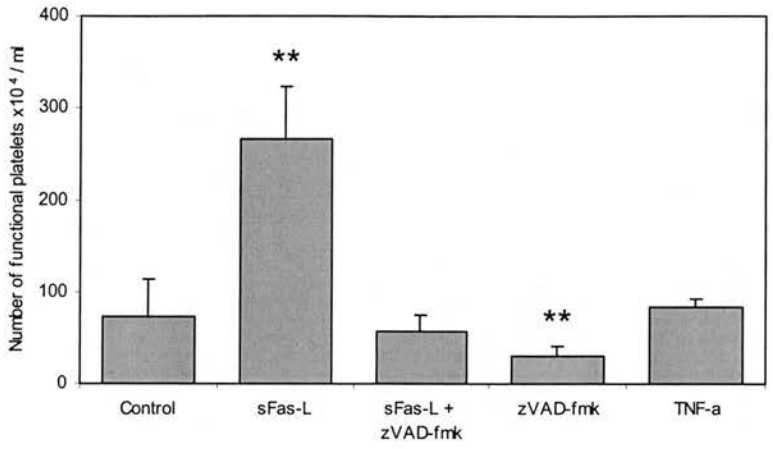


Figure 5.11: Production of functional platelets can be significantly augmented by ligation of the Fas death receptor with soluble Fas ligand. Purified mature MEG-01 MKs were cultured for 18 h with reagents as indicated, and culture supernatants separated and assayed by flow cytometry for the yield of functional platelets, as detailed in Figure 5.3. Production of functional platelets was significantly increased with human recombinant sFas-L, which was revealed to be caspase-dependent by the inhibitory effect of zVAD-fmk. Interestingly, ligation of the TNF death receptor did not promote platelet production. Data represent mean ± one S.D. of n = 3. ** represents $p < 0.02$ compared to control.

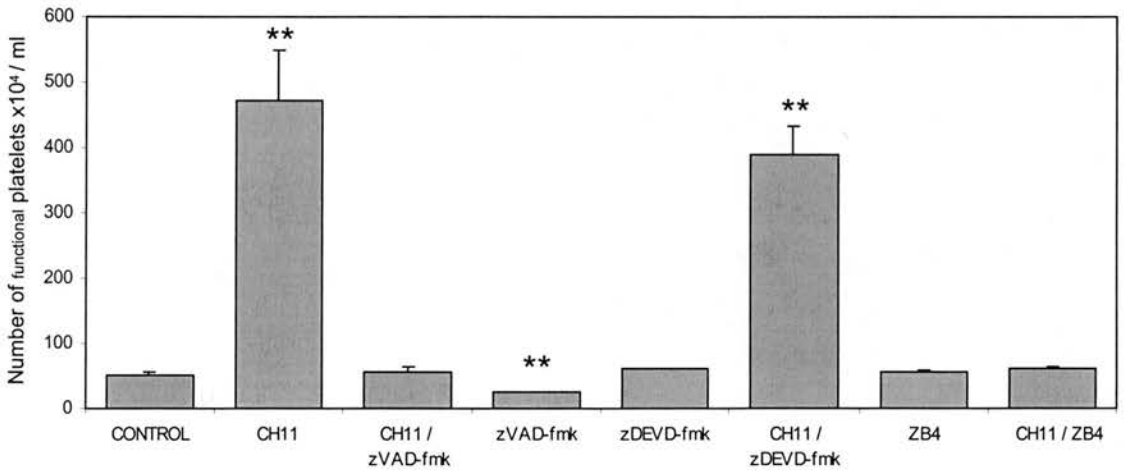


Figure 5.12: Production of functional platelets can be significantly augmented by ligation of the Fas death receptor with anti-Fas mAb clone CH.11. Purified mature MEG-01 MKs were cultured for 18 h with reagents as indicated, culture supernatants separated and assayed by flow cytometry for the yield of functional platelets as detailed in Figure 5.3. Production of functional platelets was significantly increased by the anti-Fas agonistic mAb CH.11, again revealed to be caspase dependent by the inhibitory effect of zVAD-fmk. Again the caspase-3 inhibitor DEVD-fmk had no effect on constitutive or induced production. Reassuringly, the anti-Fas antagonistic mAb ZB4 was able to block the effect of CH.11, indicative of the effect of CH.11 to be specifically ligating Fas. Data represent mean ± one S.D. of n = 4. ** represents $p < 0.01$ compared to control.

conversely inhibition of constitutive production occurs using zVAD-fmk, active caspases must be strongly implicated to play a pivotal role in platelet formation.

5.5 The physiological relevance of observations in MK cell lines is supported by two primary systems

The cloning and subsequent availability of the recombinant MK growth and differentiation factor thrombopoietin (TPO) has presented researchers with the opportunity to expand and culture primary MKs *in vitro,* leading to full terminal differentiation and platelet release (deSauvage *et al* 1994; Lok *et al* 1994). Using murine femoral bone marrow cells cultured in the presence of human TPO, mature MKs can be obtained within 5 days. In addition, and in collaboration with Prof. David Jones, we were able to investigate platelet production within a recently developed novel *ex vivo* bone core bioreactor system named the Zetos™.

5.5.1 Primary murine MKs differentiated in culture respond comparable to cell lines

Following five days of ex-vivo expansion and differentiation in the presence of TPO, primary murine MKs were assayed for the lineage specific fibrinogen receptor (CD41/61) before and after enrichment through a discontinuous BSA gradient. By flow cytometry the unpurified MK population exhibits a large heterogeneity in its SSC profile, caused by the increasing granule content as cells mature. Staining of these cells revealed MKs with a greater SSC profile, and hence greater internal granule content, expressed higher levels of CD41/61, and in the expected 1:1 ratio as evidenced by the fluorescent profile on a dot plot occurring as x = y (Figure 5.13). Likewise, cells with lower SSC profiles progressively expressed lower levels of CD41/61. Following enrichment, around 98% of MKs display the higher SSC profile and express high levels of CD41/61, indicative of mature MKs (Figure 5.13). Reassuringly, the ability of zVAD-fmk to inhibit constitutive platelet production was also observed for these mature primary murine megakaryocytes, significantly inhibiting around 50% of production. Conversely, following 18 h of treatment with Jo-2, a murine Fas agonistic mAb

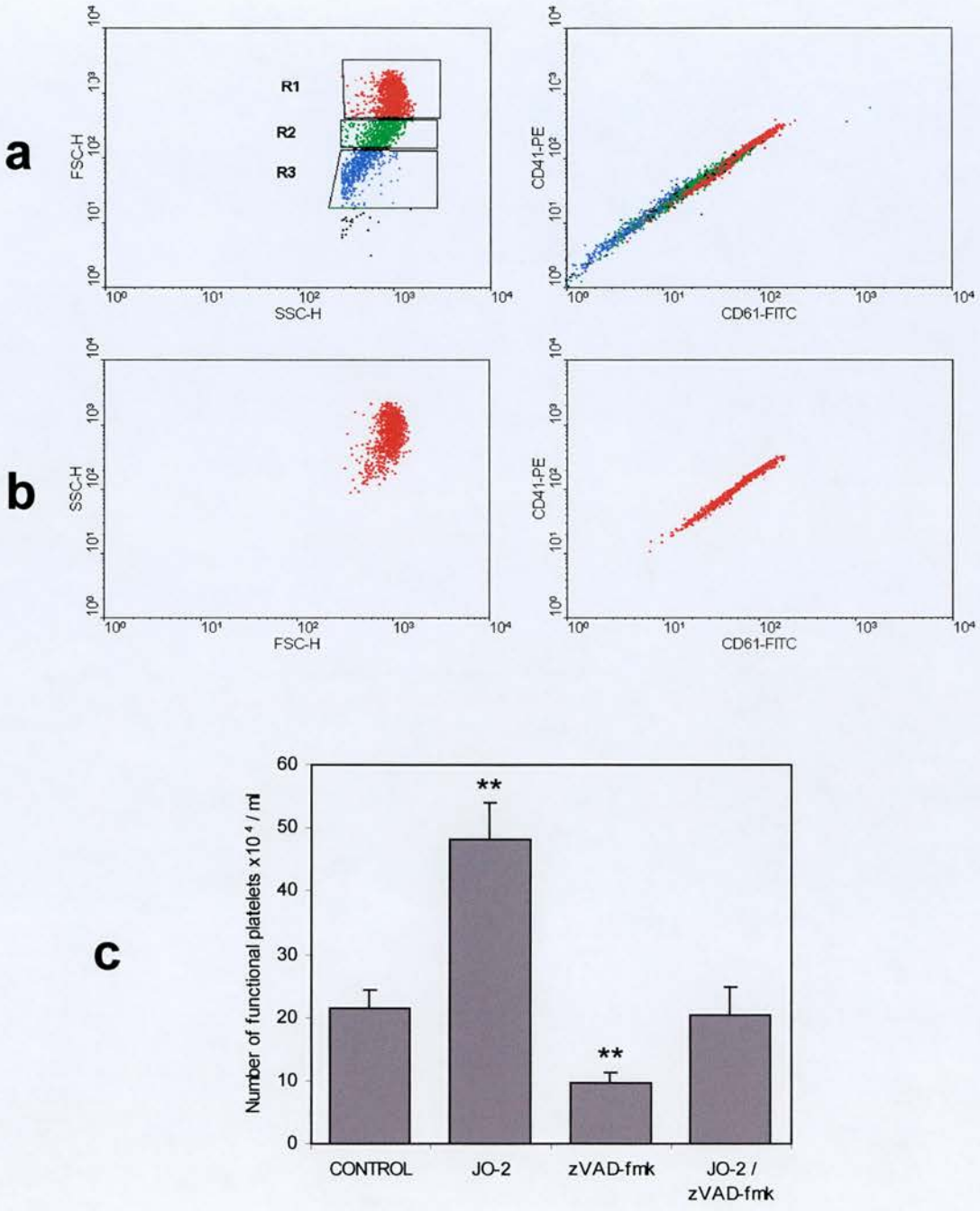


Figure 5.13: Caspase-dependent production of platelets by primary murine MKs is augmented by Fas ligation. a, Primary murine bone marrow cells cultured in the presence of TPO for 5 days were analysed by flow cytometry for CD41/61 expression. A mixed population of cells is evidenced with varying levels of CD41/61 expression. **b,** Following purification through a discontinuous BSA gradient, all retained cells can be seen to express high levels of CD41/61, indicative of mature MKs. **c,** Purified mature primary murine MKs produce functional platelets, inhibitable with zVAD-fmk and augmented with the anti-murine Fas ligating antibody JO-2. Functional platelets were enumerated by flow cytometry based on response to thrombin stimulation as detailed previously. Data represent mean ± one S.D. of n = 4. ** represents $p < 0.02$.

equivalent to CH.11, we observed that the number of functional platelets produced increased, again revealed to be caspase-dependent by the inhibitory effects of zVAD-fmk (Figure 5.13).

5.5.2 An Ex-vivo bone culture system recapitulates data seen in vitro with MK cell lines

Representing a new and novel extension of organ culture systems, the Zetos™ represents a system in which machined bone cores, obtained typically from femoral heads during hip surgery, can be kept alive in excess of 48 days within a perfusion system. Originally developed to study the effects of loading conditions on bone reabsorption and reconstruction, and with particular emphasis on investigating bone atrophy during spaceflight, in collaboration with the European Space Agency, the system had not previously been studied for its potential use in haematopoiesis. Human trabecular bone from femoral heads was found to constitutively produce PLPs that stained positive for the lineage specific fibrinogen receptor (CD41/CD61) (Figure 5.14). Constitutive PLP production over an 18 h period was inhibited robustly by the poly-caspase inhibitor zVAD-fmk, and could be augmented with low levels of the human Fas agonistic antibody CH.11, again blockable with zVAD-fmk (Figure 5.14). Although the number of PLPs generated precluded any functional assessment, ultrastructural analysis of perfused media components by TEM showed the presence of ~2μm bodies with a morphology identical to that observed for circulating blood platelets (Figure 5.14). In particular, TEM demonstrated the presence of an extended and enlarged canalicular system, a typical distribution of alpha and dense granules, and small pseudopodia extensions (possibly induced by flow within the system), as indicative of *bona fide* functional platelets. Although the platelet data only represents an initial investigation, further work appears to suggest that an intact haemopoietic stem cell system remains viable over more prolonged culture periods. TEM of culture supernatants collected following 5 d of bone core culture appear to contain mononuclear leukocyte-like cells, which could potentially be monocytes, eosinophils or neutrophils (Figure 5.14). Given the relatively low lifespan of blood leukocytes this may implicate a recent formation, and hence by inference the presence of myeloid stem cells.

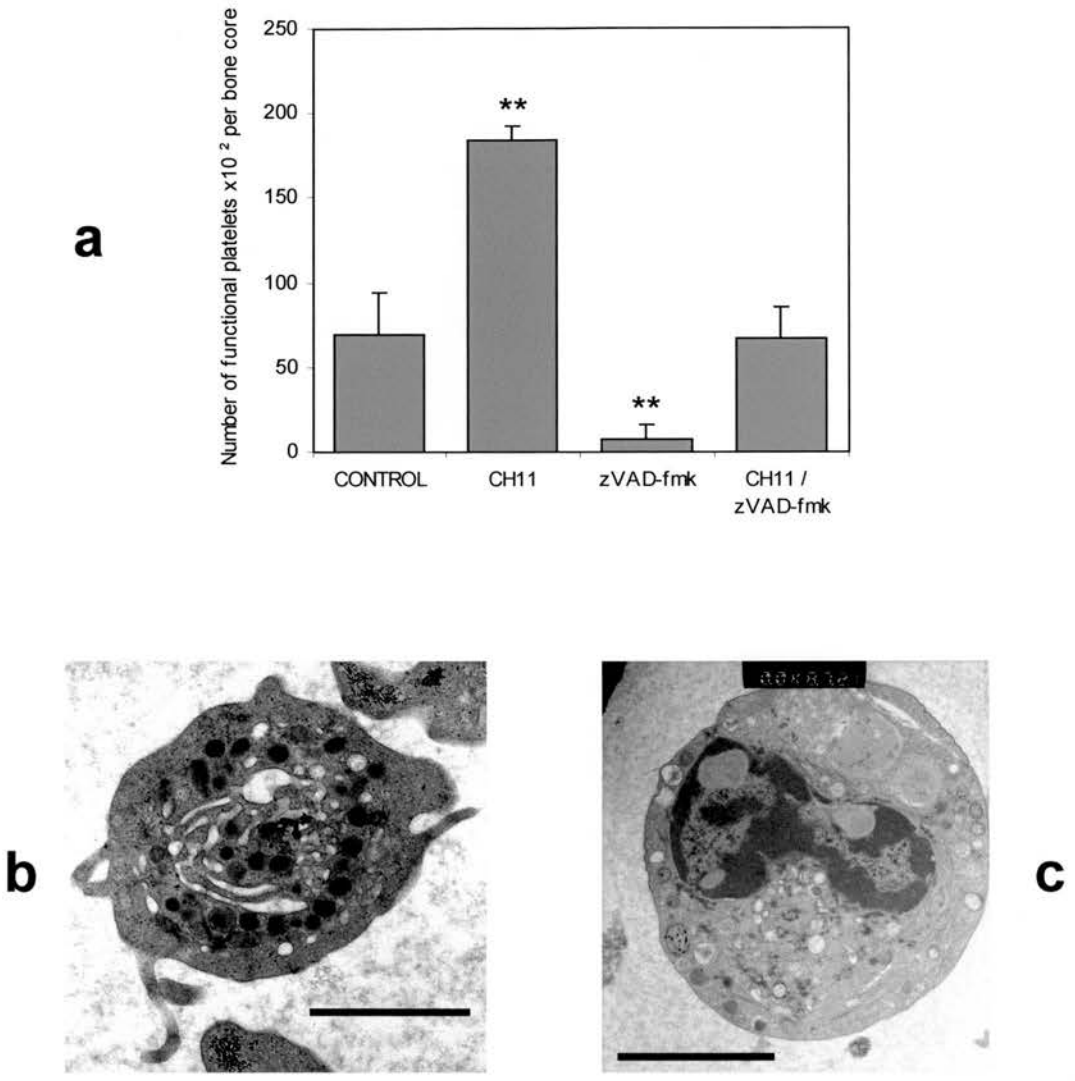


Figure 5.14: Caspase-dependent production of platelets by human bone cores is augmented by Fas ligation. a, *Ex-vivo* human bone core ex-plants maintained within a Zetos bone perfusion chamber were exposed to fresh media containing CH.11 and/or zVAD-fmk as indicated. Perfused media 'post bone core' was collected over 18 h, and CD41/61 platelets enumerated by flow cytometry. Data represent means ± one S.D. In some experiments perfused media components were prepared for TEM, and showed the presence of platelet-like bodies **(b)**, scale bar represents 1 μm, and leukocyte-like nucleated cells **(c)**, scale bar represents 5 μm.

5.6 A morphological analysis of MKs reveals apoptosis to occur actively during platelet formation

Although the previous data suggest that MK cell death strongly correlates with functional platelet formation, it gives no insight into what is actually happening to the cells on a more "individual" basis. Therefore, to address the nature of the modulation caspase inhibition or induction appeared to be playing we turned to TEM, phase, and epifluorescent microscopy to further explore whether apoptosis of megakaryocytes was causal and not consequential to platelet production.

5.6.1 MEG-01 MKs mature to large cells containing ultrastructural features identical to those observed for primary MKs

During the terminal stages of maturation MKs undergo rounds of endomitotic polyploidisation to greatly increase cell volume, whilst synthesis of numerous alpha and dense granules begins, ready for conferment to the new platelets. In addition, an extensive system of internal membranes develops, termed demarcation membranes, which were originally believed to mark out fields or territories of new platelets, but are more likely to serve as reservoirs for the large amounts of membrane required to "package" platelets. By light microscopy we observed that untreated primary murine and human megakaryocytic cell lines matured over 5 days from small 10-20μm cells to larger 40 μm cells with a smooth appearance by phase contrast, and which could be readily enriched using a discontinuous BSA gradient (Figure 5.15). Reassuringly, TEM analysis of BSA purified mature MEG-01 MKs revealed the majority of cells to contain numerous alpha and dense granules (Figure 5.15). Furthermore, these cells containing granules displayed distinct evidence of an extensive demarcation system (Figure 5.15). Previous reports had claimed the MEG-01 cell line did not mature to produce granules and was unable to develop demarcation membranes, a conclusion clearly at odds with our cells under the conditions employed. Also noteworthy is the evenly dispersed heterochromatin within the polylobed nucleus seen in this section (Figure 5.15). In addition, analysis of culture supernatants by TEM revealed numerous platelet-sized bodies that contained dense and alpha granules, in line with that regularly seen for blood platelets (Figure 5.15). Interestingly, a subset

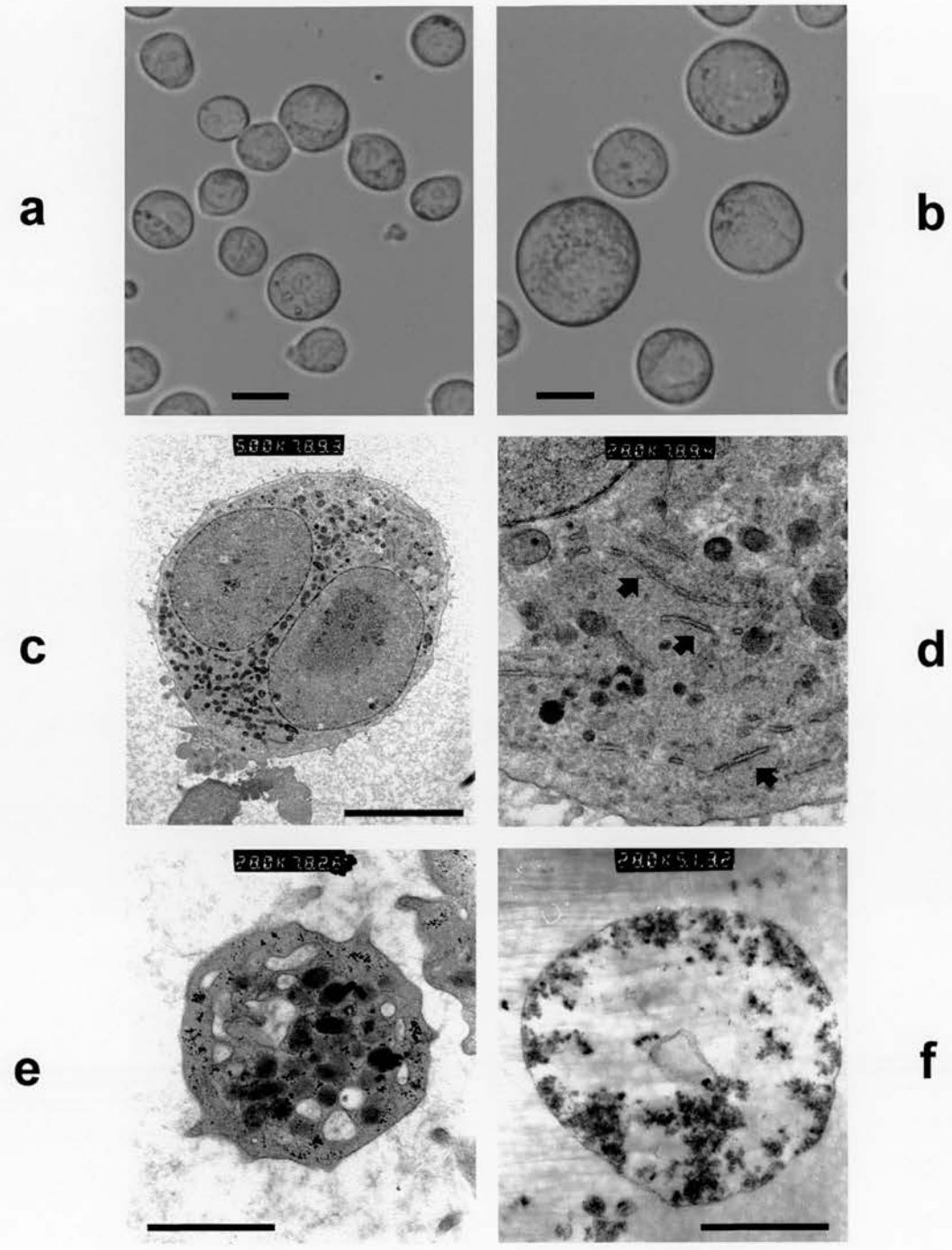


Figure 5.15: Mature MEG-01 MKs contain ultrastructural features identical to that reported for primary MKs, and produce platelets with a morphology indistinguishable from blood platelets. a, MEG-01 MKs typically appear as 20μm cells with a smooth appearance by phase. **b,** In culture MKs constitutively mature to larger 40μm cells, which can be enriched for by using a discontinuous BSA gradient. **c,** TEM of a purified mature MEG-01 MK, shown at higher magnification **(d)**, demonstrates a typical distribution of dense and α-granules, displays a characteristic demarcation membrane system (arrows), and contain nuclei with evenly dispersed heterochromatin. **e,** Spontaneously in culture MEG-01 MKs produce functional platelets with a morphology by TEM indistinguishable from blood platelets. **f,** In addition, a subset of platelet-sized bodies exist with a morphology akin to the senescent platelets reported in Chapter 3. Scale bars represent 20μm, except **(e, f)** which represent 1 μm.

of platelet-sized bodies existed, but at a lower frequency than "normal platelets", with morphology consistent with that reported in chapter 3 for the senescent platelets. Given the culture period of 18 h before supernatant collection, platelets formed early in this period may undergo death, especially given the absence of human plasma-derived survival factors (Figure 5.15).

5.6.2 A subpopulation of mature megakaryocytes contain cytoplasmically active caspases

In contrast to the normal smooth appearance by phase microscopy of the large mature MKs displayed above (Figure 5.15), a distinct subpopulation was observed which exhibited a more contoured appearance (Figure 5.16). Staining of live purified mature MEG-01 MKs with the fluorescent poly-caspase inhibitor zVAD-FAM (CaspaTag), which binds active caspases, and analysis by confocal microscopy revealed the contoured subpopulation to display a broad pattern of cytoplasmic staining. Interestingly, Z-axis scans revealed the nucleus to remain caspase negative (Figure 5.16; arrow). However, as the CaspaTag reagent is based on the zVAD-fmk peptide, and thus acts as a competitive inhibitor, the reagent is effectively only of use to display a "snapshot" of the caspase activity within the cell at the time of staining. Given this, it was impossible to perform a time course analysis to reveal whether this phenotype progressed to a proplatelet bearing form.

5.6.3 Proplatelet bearing MKs contain active caspases and show evidence of nuclear condensation

Production of platelets has been universally accepted to occur through intermediate structures termed proplatelets, consisting of thin cytoplasmic processes along which platelet sized nodes form. Again using the CaspaTag reagent, active caspases were detected within the MKs bearing proplatelet extensions, in which the fluorescence appeared to remain localised to the main cell body (Figure 5.17). In addition, dual staining with CaspaTag and the nuclear binding Hoechst 33342 revealed that proplatelet bearing MKs failed to exclude the vital dye, and clearly displayed nuclear condensation and fragmentation (Figure 5.17). Hoechst staining also revealed the nuclear material to remain confined within the cell body, in contrast to other cell types, e.g. fibroblasts, which undergo typical

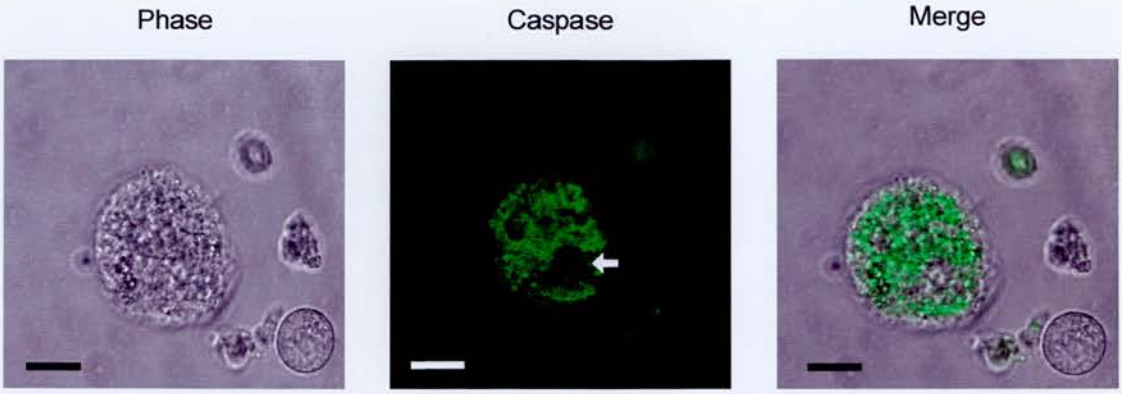


Figure 5.16: A subset of large mature MKs display a contoured morphology and contain cytoplasmically active caspases. Purified mature MEG-01 MKs were stained with the poly active-caspase specific substrate CaspaTag. On examination by confocal microscopy a subset of large MKs can be seen to display a contoured appearance by phase, in contrast to the normal smooth appearance seen in Figure 5.16. This subset also stained for active caspases within the cytoplasm, but interestingly the nucleus remained negative (arrow). Scale bars represent 20 μm.

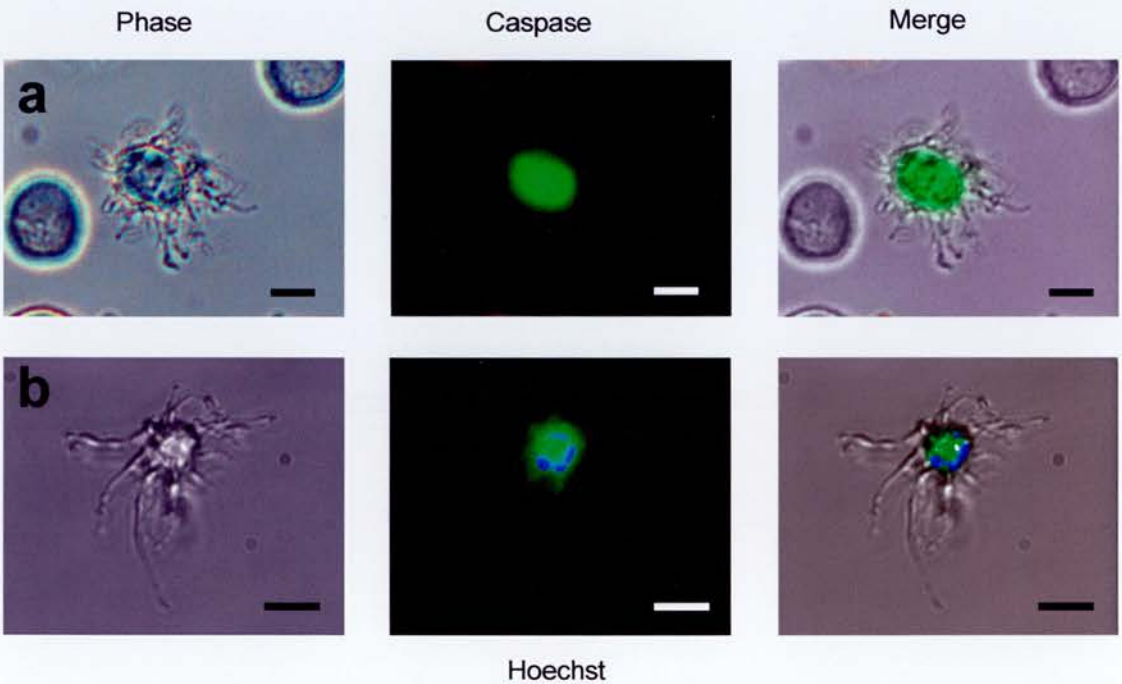


Figure 5.17: Proplatelet extending MKs exhibit nuclear pyknosis and contain active caspases. a, A mature MEG-01 MK displaying proplatelet extensions stained positive for active caspases (green), which remained localised within the main cell body. Note that MKs without proplatelet extensions (left and right) lacked caspase activity. **b,** Dual staining of nuclear material with Hoechst 33342 (blue), and for active caspases reveals MKs bearing proplatelets to contain pyknotic nuclei, with nuclear material remaining within the cell body along with active caspases. Scale bars represent 20 μm.

blebbing morphology and whereby nuclear material localises throughout the apoptotic bodies (Coleman *et al* 2001; Sebbagh *et al* 2001).

5.6.4 TEM of mature MKs extends evidence of apoptosis occurring during platelet formation

In support of proplatelet extension occurring concurrent with nuclear condensation, TEMs of MKs with a typical demarcation membrane system, apparent throughout the cytoplasm, show evenly dispersed nuclear material inconsistent with apoptosis (Figure 5.15). In contrast, MKs displaying extensive "striations" below the cell membrane, indicative of the very earliest stages of cytoplasmic projection, exhibited the clear nuclear condensation typical of early apoptosis (Figure 5.18). These features indicate major cytoskeletal and membrane rearrangements within the cell before proplatelet extension is witnessed, and essentially appear as "unravelled" proplatelets, with size and structure in-line with that observed by others (Italiano *et al* 1999). Whether these internal changes could cause the visual change from a smooth appearance by phase to the more contoured phenotype described above is undetermined. In addition, MKs seen to display proplatelet extensions appeared to have proceeded to more extensive nuclear condensation and fragmentation (Figure 5.18). Although few proplatelet processes appear in this particular section, the typical 40μm size of mature MKs and 60nm ultrathin sections cut would require around 700 serial sections to be taken in order to obtain a TEM with more proplatelets within a section. In retrospect, clear evidence of nuclear condensation has been presented in many previous ultrastructural studies of MKs actively producing platelets (Choi *et al* 1995; Cramer *et* al 1997; Zauli *et al* 1997), but the authors overlooked any relevance to apoptosis.

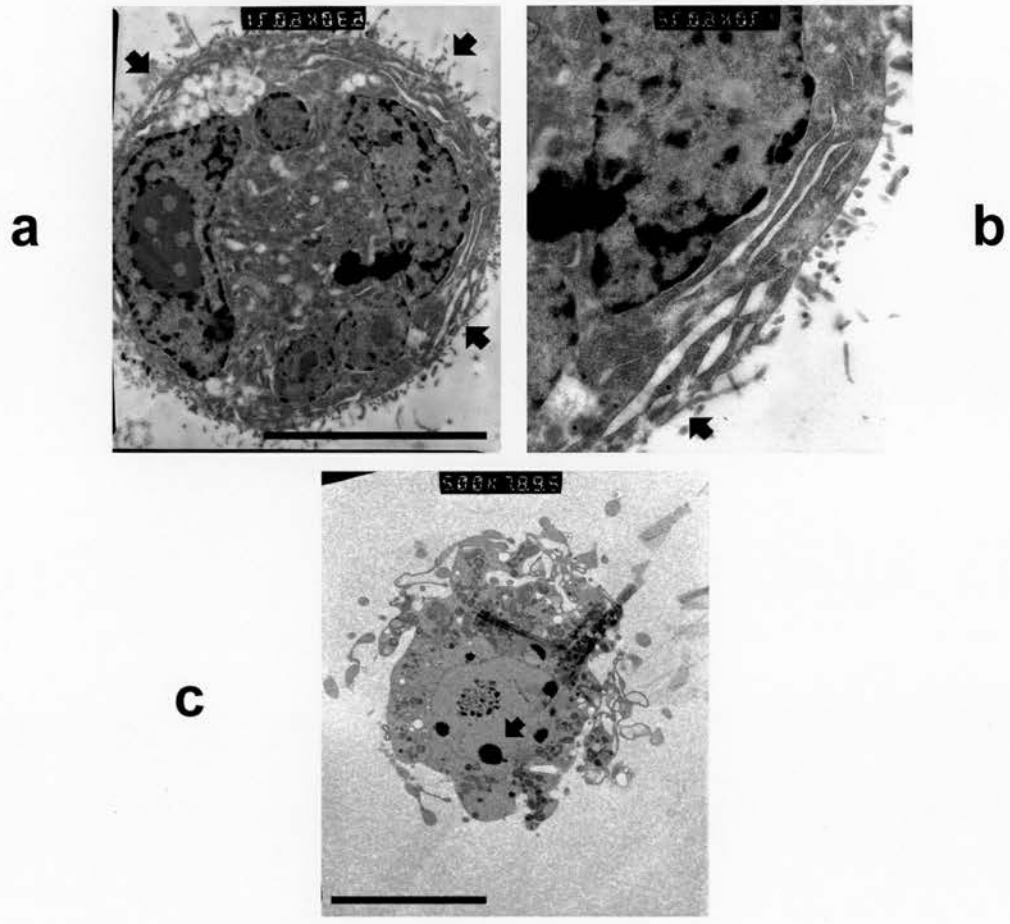
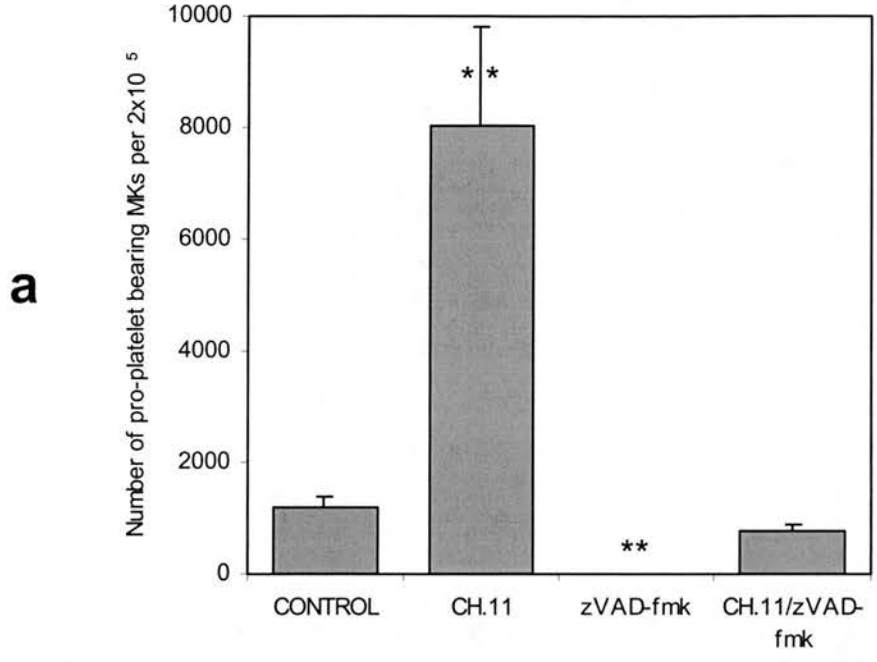


Figure 5.18: MKs undergoing proplatelet extension display nuclear condensation and fragmentation typical of apoptosis. Purified mature MEG-01 MKs where examined by TEM. **a,** A MK exhibiting early cytoplasmic rearrangements consistent with platelet formation (between arrows), contained a nucleus displaying heterochromatin condensation typical of early apoptosis, shown at higher magnification in **(b)**. **c,** A MK cell bearing proplatelets shows extensive condensation and fragmentation of the nuclear material (arrow). All scale bars represent 20 μm.

5.6.5 Proplatelet formation can be modulated by induction of inhibition of caspases

Given the significant effect caspase inhibition or Fas treatment had on the total yield of platelets, and the clear evidence of caspase activation and apoptotic morphology occurring during proplatelet extension, we investigated if the total number of proplatelet bearing MKs was directly modulated by the reagents. Quantification by light and epifluorescence microscopy of treated cells confirmed that CH.11 and zVAD-fmk modulated the formation of proplatelet bearing MKs, in which 89 ± 3% (mean ± S.D. n = 3) of these cells stained positive for active caspases, and 95 ± 3% (mean ± S.D. n = 3) showed signs of nuclear pyknosis (Figure 5.19). In contrast to previous experiments where zVAD-fmk reduced total platelet production by around 50%, the effect of the caspase inhibitor in these experiments resulted in almost total loss of the proplatelet bearing phenotype. However, these experiments were counted after an 8 h time course, in comparison to an 18 h culture for total platelet yield, and may reflect instability of the small peptide inhibitor, which is well known to have a short half-life and be sensitive to hydrolysis. These results strongly suggest that constitutively, or in response to Fas ligation, mature megakaryocytes activate caspases and proceed to a proplatelet bearing form in which nuclear condensation is observed.



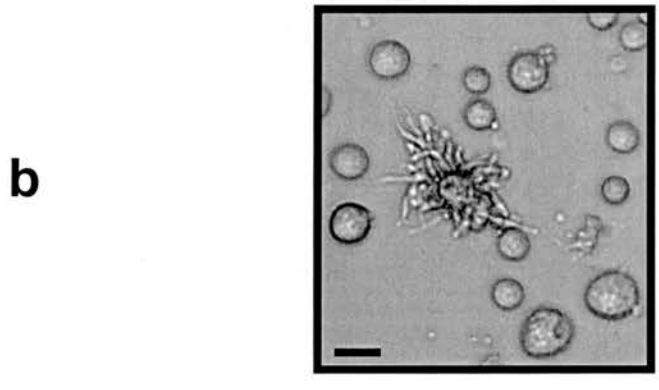


Figure 5.19: The number of proplatelet bearing MKs can be modulated through induction or inhibition of caspases. Purified mature MEG-01 MKs were culture for 8 h in the presence of reagents as indicated, and formaldehyde added to a final concentration of 0.5% to stabilise processes. **a,** Entire wells were counted blind by phase microscopy for the total number of pro-platelet bearing MKs. Data represent mean ± one S.D. of n = 3. ** represents $p < 0.0001$ compared to control. **b,** A representative example of typical proplatelet bearing MK morphology as examined by phase microscopy. Scale bar represents 20 μm.

5.7 MK proplatelets maintain their ΔΨM during constitutive and induced death

Death receptor pathways typically proceed through activation of effector caspases, such as 8 or 10, leading to Bid cleavage and subsequent mitochondrial insertion resulting in loss of $_{\Delta}\psi M$, release of cytochrome-C, and eventual caspase-3 activation. Platelets are known to utilise oxidative phosphorylation (Doery *et al* 1970) and we have previously demonstrated a loss of $_{\Delta}\psi M$ and release of cytochrome-C to occur as an early event in blood platelet programmed cell death (section 4.2.1). If MK apoptosis proceeded via a mitochondrial-dependent pathway, it would require newly formed platelets to re-establish their $_{\Delta}\psi M$. As this seemed implausible we investigated the involvement of the mitochondria.

5.7.1 Caspase-dependent platelet production occurs without loss of $\Delta\psi M$

Mature MEG-01 MKs were cultured under control conditions or with CH.11 for 8 h, followed by staining with the inner-mitochondrial membrane potential sensitive dye JC-1 (Salvioli *et al* 1997). Functional mitochondria can be seen localised within platelet-sized nodes along the extended proplatelets, as evidenced by orange fluorescence, whether induced with CH.11, or cultured under control conditions (Figure 5.20). Intriguingly, 94 ± 3% (mean ± S.D. n = 3) of proplatelet bearing MKs with an intact $_{\Delta}\psi M$ showed evidence of nuclear condensation on dual staining with Hoechst 33342 (Figure 5.20). Analysis by confocal microscopy revealed residual mitochondria to be polarised within the cell body to the MK edge with the remaining proplatelet "bridge" still attached (Figure 5.20; arrow). This evidence supports the hypothesis by Italiano *et al* (1999) that platelet specific material and organelles are actively delivered to the forming platelet tip, most likely by attachment to open microtubule loops, and that platelets are not present as nascent pre-formed entities within MK demarcation membranes. Therefore, extraordinarily, it seems that caspase-dependent proplatelet extension, nuclear condensation, and eventual functional platelet release occurs with an intact mitochondrial membrane potential.

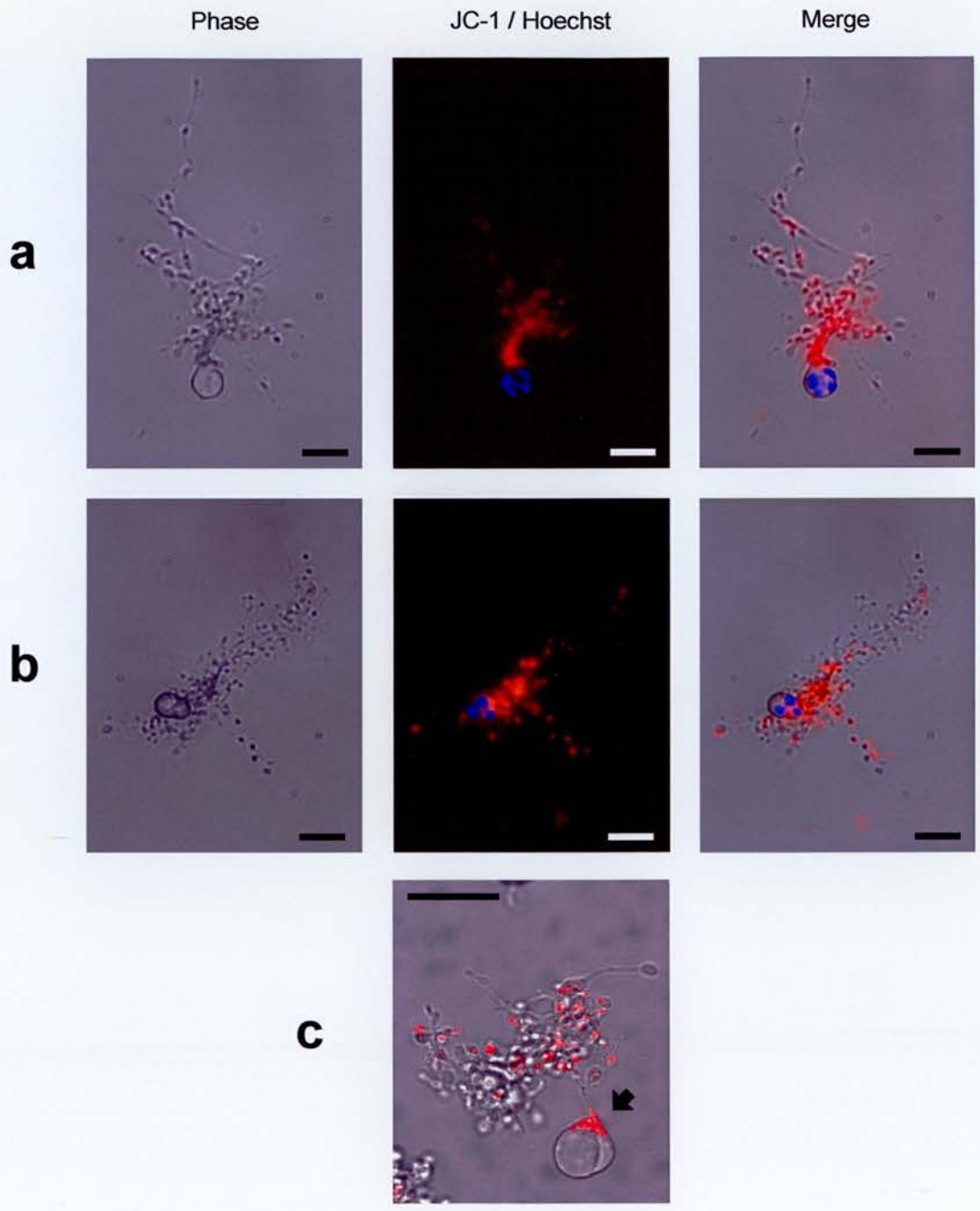


Figure 5.20: Functional platelet production is associated with the maintenance of an inner-mitochondrial membrane potential. a, b, Mature MEG-01 MKs cultured for 8 h untreated **(a)** or with CH.11 **(b)** were double stained with the inner mitochondrial membrane potential sensitive dye JC-1 (orange), and the nuclear staining vital dye Hoechst 33342 (blue). Mitochondria with an intact $\Delta\psi M$ are seen localised within platelet-sized nodes along extended proplatelets, whilst the cell bodies simultaneously display nuclear condensation. **c,** On examination of JC-1 stained MKs by confocal microscopy, with limited Z-axis scanning due to cell and pro-platelet motility, mitochondria with an intact $\Delta\psi M$ can be seen polarised within the cell body toward the remaining attached pro-platelet (arrow). All scale bars represent 20 μm.

5.7.2 MEG-01 MKs contain BID which is truncated in response to Fas ligation

The BH3 only domain pro-apoptotic protein Bid has been shown to be a specific proximal substrate of caspase-8 in the Fas apoptotic signalling pathway. Full length Bid (26kDa) remains localised in the cytoplasm, but following cleavage by caspase-8 the BH3 containing COOH-terminal (15kDa) translocates to mitochondria, triggering cytochrome-C release (Luo *et al* 1998; Li *et* al 1998). Given the increased apoptosis and platelet production by MKs on Fas ligation, and the concomitant maintenance of the $\Delta\psi M$, we investigated whether a defect in the Bid pathway could be mediating this novel phenomenon. Western-blot analysis using an anti-Bid pAb recognising both the full length and truncated active form revealed purified mature and immature MKs to contain Bid at comparable levels to Jurkats, used as a positive control (Figure 5.21). Treatment of MKs with CH.11 prior to sample analysis revealed Bid to be truncated to the active form, again with comparable amounts processed by mature and immature MKs, or Jurkats (Figure 5.21). Therefore, maturation of MKs does not confer a resistance or inability to process Bid, and hence prevent $\Delta\psi M$ loss. However, inhibition of truncated Bid before mitochondrial disruption, particularly by members of the Bcl-2 family, still present a potential explanation. In addition, the somewhat basic division of MKs with a BSA gradient into mature and immature gives no insight into whether the proplatelet bearing cells have processed Bid.

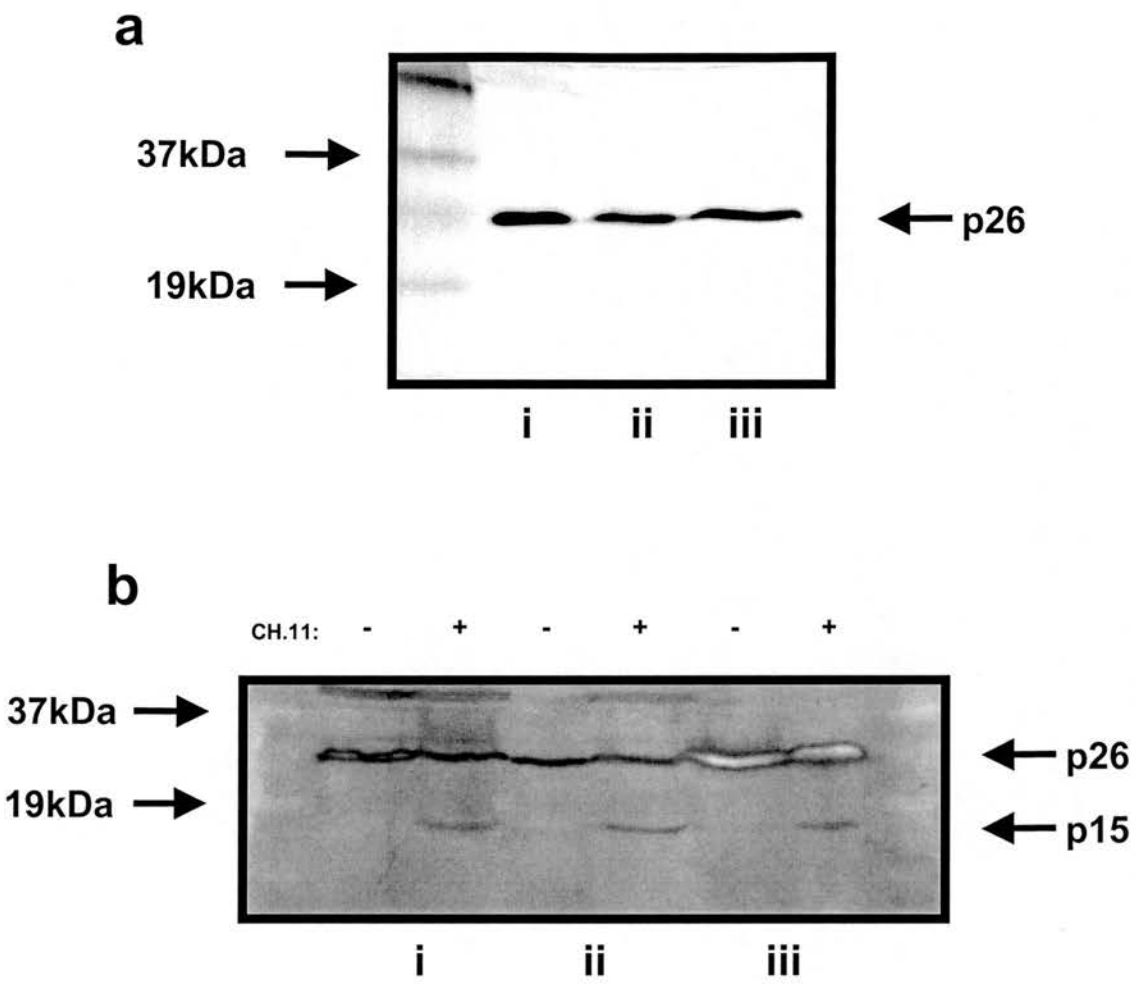


Figure 5.21: MEG-01 MKs contain Bid which is truncated in response to Fas ligation. a, Whole cell lysates prepared from Jurkats **(i)**, immature MEG-01 MKs **(ii)**, and mature MEG-01 MKs **(iii)** were analysed by Western-blot for expression of Bid with a pAb recognising the 26kDa full length and 15kDa truncated form. All cells contained comparable amounts of full length Bid, based on even protein loading, with no change as MKs mature. **b,** Whole cell lysates were prepared from the same cell types, but with or without prior CH.11 treatment. Truncated Bid was evidenced in all cells exposed to CH.11, suggesting the maintenance of $\Delta\Psi M$ is not mediated through an inability to truncate Bid by MKs mature.

Discussion

The key conclusion of this work is that a form of compartmentalised apoptosis of a progenitor cell is a hitherto unrecognised mechanism for generation of multiple, functional anucleate daughter cells. Thus, proplatelet bearing MKs exhibited clear morphological evidence of nuclear changes of apoptosis and caspase activation in the main cell body, but bore processes that retained mitochondrial membrane potential and yielded functional anucleate progeny (platelets) that were not marked for immediate clearance by macrophages. Caspase inhibition reduced platelet production, whilst ligation of MK Fas increased platelet production in a caspase-dependent manner, supporting caspase-directed MK apoptosis as a mechanism for production of viable platelets. However, the "compartmentalised" nature of such apoptosis was further emphasised by our previous finding that caspase-9, a key component of the apoptosome following mitochondrial cytochrome-C release, is present in progenitor MKs but is excluded from platelets, accounting for the caspase-independent nature of constitutive platelet death.

Although our findings differ, they do share some parallels with the probable early role of caspases in generation of the erythrocyte, another anucleate cell. Rather than production of multiple anucleate daughter cells from a single progenitor (Stenberg and Levin 1989; Italiano *et al* 1999), the data suggest that viable erythrocytes transiently activate caspases, resulting in cleavage of a subset of structural proteins that may help lead to the anucleation of the erythroblast (Zermati *et al* 2001). The erythroblast then continues maturation to produce a single anucleate erythrocyte (Gregory and Eaves 1978). However, this system reassuringly demonstrates that the transient activation of caspases is able to selectively cleave structural proteins such as acinus and laminins, whilst failing to cleave others such as DFF45/ICAD and GATA-1. Although this selectivity remains unexplained within the erythroblast system, it further adds support to our suggestion that the apoptotic machinery can be compartmentalised. Clearly, further work will be required to define the mechanisms by which caspase activation in one part of the cell can lead to nuclear changes, whilst mitochondria in a different area of the cell retain their transmembrane potential. Similarly our data also reinforce the concept that plasma membrane changes of apoptosis may be dissociated from the caspase-directed program of nuclear

condensation and fragmentation (Knepper-Nicolai *et al* 1998; Harper *et al* 2001). Nevertheless, as we have emphasised, platelet production from MKs is a complex active mechanism resulting in the specific delivery of progenitor cell cytoplasmic components into the proplatelets. Thus, it is possible that there is specific exclusion of death pathway components upstream of mitochondria, analogous to the exclusion of caspase-9 suggested by our data. Unfortunately, many possibilities could not be tested directly since platelet yields from MK cultures were insufficient for blotting studies, and the delicate nature of proplatelet-bearing MKs, which tended to lose their processes on manipulation, excluded direct immunofluorescent localisation of caspase-9. Nevertheless, it seems clear that evolution has achieved adaptations of the basic program of cell death by apoptosis to produce anucleate cells of critical importance in blood.

The maintenance of $\Delta\psi M$ within proplatelet extensions whilst the cell body displays nuclear condensation is fascinating. Initiation of apoptosis through death receptors, the extrinsic pathway, is often presented within the literature to be a mitochondrial-independent process. This has tended to be based on the evidence that in some cell systems Fas death cannot be blocked by Bcl-2, and hence is not mediated through the mitochondria (Scaffidi *et al* 1998). However, this "designation" only refers to the initial activation of a caspase, which in the case of caspase-8 and its subsequent activation of caspase-3, obviously occurs without any mitochondrial involvement. Nevertheless, it is well established that active caspase-8 cleaves the BH3 only protein Bid, the truncated form of which is thought to insert into the mitochondrial membrane to mediate release of cytochrome-C (Li *et al* 1998; Luo *et* al 1998). This does not appear to be happening in our system given the $\Delta\psi M$ is always maintained in proplatelet bearing MKs. Although the importance of mitochondrial permeability transition (PT) in initiating apoptosis remains highly controversial, even staunch opponents of the theory admit, and have published, that loss of $\Delta\psi M$ does inevitably occur during apoptosis. Principally, "$\Delta\psi M$ was unchanged in cells treated with anti-Fas antibody for ~2.5 h, but dropped precipitously at ~3 h after treatment" (Matsuyama *et al* 2000), concluding that although PT is an event occurring after cytochrome-C release it will inevitably occur. We clearly cannot have cytochrome-C release given that platelets utilise oxidative phosphorylation during their lifespan of ~8 days, and subsequently release cytochrome-C during their own constitutive death (section 4.2.2).

Interestingly, some cell types have been demonstrated to be protected against death receptor induced apoptosis by Bcl-2, suggesting a requirement for the secondary caspase amplification that Bid-mediated cytochrome-C release would give. However, deficiencies in Bid, but not the presumed initiator caspase of the Bid pathway, caspase-9, protect against Fas induced death (Zheng *et al* 2000), implying that Bid is capable of mediating some as yet unknown pathway. In our opinion the data could suggest the main objective of Bid-mediated mitochondrial disruption is the release of caspase inhibitor antagonists such as Smac/Diablo. This may potentially explaining the disparity in Bcl-2 protection against Fas death, with cell types containing higher levels of the endogenous caspase inhibitor XIAP requiring the Bid pathway-induced mitochondrial release of antagonist. It is not clear whether the Bcl-2/Bcl-x_L antagonism of Bid is through binding of the proform to prevent cleavage, sequestration of the active truncated form, or interaction at the mitochondrial membrane to prevent Bid-mediated release, mainly due to varying results dependent on the presence or absence of detergents. Whatever mechanism is correct, expression of Bcl-x_L has been demonstrated to inhibit all of the apoptotic phenotypes induced by tBid (Li *et al* 1998). Interestingly, on maturation MKs have been demonstrated to upregulate their levels of Bcl-x_L by up to ten-fold (Terui *et al* 1998; Sanz *et al* 2001). Intriguingly, remnant denuded MKs have been demonstrated to contain no Bcl-x_L, whilst platelet and proplatelet-like fragments within the same culture supernatants, along with fresh blood platelets, have been shown to be Bcl-x_L positive (Sanz *et al* 2001). Similarly, MKs are known to contain typical amounts of Bcl-2, however, we and others have demonstrated that platelets do not contain Bcl-2 (Terui *et al* 1998). This again strongly supports the idea that MKs are capable of the compartmentalisation of cellular components, and given the structural and functional similarity of the Bcl-2 and Bcl-x_L proteins, this appears to be an extremely specific process. Speculatively, given that our mature MKs (albeit a rough fractionation of the MKs, and not a pure population of proplatelet bearing cells) appear to be capable of processing comparable amounts of Bid as Jurkats or immature MKs, could Bcl-2 be sequestering this truncated form away from the proplatelet processes to prevent cytochrome-C release and eventual loss of $\Delta\psi M$? Similarly, does the large excess of MK-derived Bcl-x_L, which is later found in platelets, stay associated with the mitochondria within the proplatelets to "safeguard" against any escaped tBid, and thus prevent loss of $\Delta\psi M$?

Fas signalling does not have to involve caspase-8, but instead can proceed through either caspase-10 (Wang *et al* 2001), -2 (Chou *et al* 1998), or RIP kinase (Holler *et al* 2000). However, caspase-2 has been shown to be highly insensitive to zVAD-fmk (Garcia-Calvo *et al* 1998), whilst RIP-kinase has been shown to induce a necrotic-like cell death without chromosomal condensation (Holler *et al* 2000), and thus both are inconsistent with our observations. Caspase-10 is the only known mammalian caspase to share homologous DEDs to caspase-8 (Fernandes-Alnemri *et al* 1996), and has been demonstrated to be able to efficiently act in the absence of caspase-8 as an apical initiator of death receptor apoptosis (Wang *et al* 2001). It has also been shown that caspase-10 has different apoptotic substrates to caspase-8, and hence may play a different role in death receptor signalling (Wang *et al* 2001). However, whether caspase-10 can cleave Bid has not been reported, and thus does not help explain our observations.

Although the combined data in this chapter strongly suggest a role for apoptosis and caspase activation in platelet genesis, any specific caspase substrates that could enable the gross morphological changes witnessed for platelet formation remain unknown. Caspases are well known to target many cytoskeletal proteins and regulators such as actin, spectrin, p21-activated kinase 2 (PAK2) and gelsolin, but most tend to result in a disruption of cellular architecture resulting in rounding of the cells, and dissociation of adhesion to the underlying matrix. However, membrane blebbing, as mentioned in the previously chapters discussion, is a caspase-directed event requiring a force generation through the actin-myosin system, and results in membrane bound bodies into which fragmented DNA has been specifically localised (Coleman *et al* 2000; Sebbagh *et al* 2000). Given that MKs potentially have the ability, like the erythrocyte example, to "limit" or moderate a more typical uncontrollable caspase cascade, could caspase cleavage of a more limited set of cytoskeletal substrates directly aid or activate regulators of cytoskeletal rearrangements to allow, for example, the actin-generated contractile force required for branching of proplatelets (Italiano *et al* 1999), or to aid in polymerisation of tubulin monomers to the base of the microtubule containing proplatelet, allowing elongation (Italiano *et al* 1999).

Protein kinase C alpha (PKCα) has been implicated to be required for proplatelet extension, based on its inhibition with pharmacological agents and isotype specific ribozymes (Rojnuckarin and Kaushansky 2001). PKCα was shown to localise to aggregates of F-actin that form at the base of proplatelet extensions, whilst PKCα inhibition prevented these aggregates form forming. Various proteins have been identified to function in actin rearrangement in association with PKC, including the myristoylated alanine-rich C-kinase substrate (MARCKS) family (Rosen *et al* 1990), pleckstrin (Ma and Abrams 1999), and the Wiskott-Aldrich syndrome proteins WASP and N-WASP (Miki *et al* 1996; 1997). Functions of PKC during apoptosis remain controversial with many reports claiming a caspase-directed activation (Ghayur *et al* 1996), whilst others suggest PKC activation inhibits cell death (Sarker *et al* 2001). The data appears to vary with the cell types, death stimuli, and PKC subspecies studied, however, the report "closest" to our system has demonstrated PKCα to be activated in a zVAD-fmk-inhibitable manner in HL-60 cells following induction of apoptosis by a range of agents (Shao *et al* 1997). However, PKC has been implicated to be able to modulate signalling from the death receptor induced DISC complex. High levels of active PKC have been shown to inhibit recruitment of FADD and hence caspase-8 to the DISC, thus preventing the caspase cascade (Gomez-Angelats and Cidlowski 2001). Speculatively, if the level of PKC activation was in a more controlled manner, could it affect signalling from the DISC to, say, prevent Bid cleavage, or to modulate the initiator or effector caspases activated, thus eliciting a different cellular response of caspase-driven proplatelet extension and apoptosis, rather than immediate "high level" caspase activation and apoptotic death?

Another fascinating outcome of this study was the opportunity to work with the bone core bioreactor system in collaboration with Prof. David Jones of the University of Marburg, Germany. His prime interest is the study of osteoblast reabsorption, and osteoclast replication of bone that is continually turned over throughout our life. Thus, a system was developed to allow the maintenance, and hence study of *ex vivo* bone core samples in the lab. However, the haematopoietic potential of the system had not been studied. As discussed in the results, production of platelets from the core replicated the data seen with the cell lines and primary MKs. In addition, the core also appeared to produce mononuclear and neutrophil-like cells ten days post surgical excision. As stated this is very

much a preliminary study, but the potential of this system for the study of general haematopoiesis is huge. A persistently highlighted weakness of more typical "culture plate" experiments is the lack of the complex matrix substratum, and three dimensional cell-cell interactions that exist within the bone marrow. Hence using the bone core a plethora of more physiological experiments could be conducted on, for example, cytokine driven differentiation, chemotherapy agents, immunosuppressants, etc, etc, without the incessant and sometimes preventable use of laboratory animals.

Essential thrombocythaemia is a myeloproliferative disease characterised by a marked alteration in megakaryocytopoiesis resulting in high levels of circulating platelets, whilst many non-immune mediated thrombocytopenias result in low circulating platelets numbers, both are of unknown aetiology. Our findings suggest that *in vivo* these abnormalities of circulating platelet numbers, of critical importance in disorders of haemostasis and in thrombotic diseases such as stroke and myocardial infarction, could reflect abnormalities in the control of MK apoptosis. Insufficient MK apoptosis, or a "non-compartmentalised" apoptosis could result in lack of platelet formation, or the direct clearance of the MKs before or during platelet formation (the logistics of MΦ phagocytosis of a mature MK would be interesting!). Some *in vivo* evidence exists to support a physiological basis for our findings. Transgenic mice over expressing the anti-apoptotic Bcl-2 (Ogilvy *et al* 1999), or ablated for the pro-apoptotic Bim (Bouillet *et al* 1999) show a 50% reduction in circulating platelets, but show no defect in MK numbers. In addition, mice deficient in components of the Fas death pathway exhibit thrombocytopenia, which has largely been attributed to autoimmune thrombocytopenia (Rieux-Laucat *et al* 1995; Le Deist *et* al 1996). Interestingly, the few TEM studies that have demonstrated MKs producing platelets *in vivo* have more often than not observed MΦ to be in the vicinity, and in some cases with processes extending around the remnant denuded nucleus (Radley and Haller 1983). Given that a recent paper has demonstrated nitric oxide (NO) to induce platelet-like particle formation through an apoptotic-like process (Battinelli *et al* 2001), and that MΦ-NO (Duffield *et al* 2000), and MΦ-Fas directed killing (Brown and Savill 1999) has been observed in many systems, could tissue MΦ within the bone marrow be "directing" the apoptosis of the MKs, and thus be ready to clear the remnant bodies and large amounts of micro vesicular debris observed during *in vitro* platelet production. Indeed, one might speculate the possibility exists that pharmacological manipulation of

MK apoptosis, and the Fas pathway in particular, might provide a novel therapeutic strategy for the management and control of thrombostasis. Furthermore, the recent clinical trials of caspase inhibitors for the clinical treatment of blood sepsis (Hotchkiss *et al* 2000), reperfusion ischemia (Cursio *et al* 1999), and neurological conditions (Robertson *et al* 2000) may reveal an unwanted side effect to be thrombocytopenia.

Chapter 6 – General Discussion

The study effectively started at "end of the line" for the MK / platelet system, looking at the ultimate changes on platelet aging and phagocytosis, worked backward to elucidate earlier potential mechanisms and effectors of platelet death, and ended with a study on the birth of platelets! Although much of this work has been discussed in the individual chapters, such discussion has tended to focus on the three studies in relative isolation. However, the combined data (in reverse order!) reveals a fascinating and seminal insight into the possibility that cell death may be a prime effector mechanism in the maintenance of thrombostasis.

To summarise, MKs differentiate and mature within the bone marrow until a terminal stage is reached whereby either constitutively, or by Fas directed killing, MKs initiate an adapted and putatively "compartmentalised" form of apoptosis. Unidentified caspases are activated resulting in membrane rearrangements and early stages of chromatin condensation. The MK extends proplatelets along with the occurrence of more extensive nuclear condensation and fragmentation. Platelet specific material is delivered through the proplatelets, including alpha/dense granules, functional mitochondria with $\Delta\psi M$ intact, Bcl-x_L, and caspase-3, all localising to the forming platelets. However, the key component of the apoptosome, caspase-9, is excluded or degraded by the MK, resulting in platelets devoid of the enzyme. Platelets are released from the proplatelet tips into circulation and typically survive for 7-10 days. Through an unknown mechanism platelets initiate their own intrinsic death program resulting in an increase in proapoptotic Bak and Bax. This is associated with a mitochondrial dysfunction that now results in loss of $\Delta\psi M$ and release of cytochrome-C. However, due to the absence of caspase-9 cytochrome-C cannot initiate the formation of the apoptosome complex, and hence subsequent activation of the effector caspase-3 does not occur. In spite of this, platelets undergo morphological and surface changes indicative of a cell death program by other uncharacterised caspase-independent mechanisms. This ultimately results in a "recognition competent" form able to be selectively removed, whereas activated or fresh platelets are not, by a range of phagocytes, including vascular endothelial derived cells, utilising a scavenger receptor-mediated mechanism.

Therefore, although of limited *in vivo* relevance, the combined data present the possibility that control of circulating platelet numbers could be maintained in part by two opposing and different cell death programs. Firstly, increased caspase-dependent cell death in MKs leads to increased platelet formation and hence release into circulation, thus raising platelet numbers. Secondly, increased caspase-independent cell death within the platelet produces a recognition competent cell able to be removed by phagocytes, hence lowering platelet number. Many, many, future experiments are needed to help understand the detailed molecular mechanisms mediating these events, and thus whether any potential for clinical intervention exists, and as such this thesis under one man power has only scratched the surface, leaving numerous "open avenues" of research potential.

From the "platelet work" we have the potential to investigate the chymotrypsin sensitive recognition molecule, or other changes on the platelet surface. This could be initiated by some simple surface biotinylations and Western blotting to attempt to identify any global surface changes on the platelet. Further investigations could use a "molecule fishing" technique, based on incubating surface biotinylated aged platelet membrane-derived microvesicles with MΦ, washing away unbound material, followed by analysis of extracted protein by Westerns with an anti-biotin Ab. Hence, any biotin positive proteins found would have been enriched for due to their binding to MΦ. Furthermore, it may prove useful to feed platelets aged for less time, and thus where a mixed population of edible/non-edible exists, to MΦ. Subsequent analysis of these MΦ by TEM may provide insight into whether only platelets aged to the vacuolated and condensed morphology reported are eaten, or whether an earlier phenotype is recognition competent. Thus, it may be possible to look for platelets with this phenotype *in vivo* in patients with conditions that may potentially have increased aged platelets, i.e. thrombocythaemias, acute splenectomy, high blood LDL. Similarly, production of monoclonal antibodies or the screening of large panels of current antibodies may identify a surface marker that is either gained or lost on aging, again allowing screening for these platelets *in vivo*, or alternatively the blocking of an epitope crucial for phagocytosis.

Much work could also be directed at the haemostatic properties of the senescent platelets. Initial work shows that mixing fresh platelets with small numbers of aged rapidly accelerates

coagulation on readdition of Ca^{2+} by around two-fold. In addition, using a novel in house developed flow system one could investigate what interaction effete platelets have with vascular endothelial cells under flow conditions, especially given that HUVECS can phagocytose them. Interestingly, a paper from Marguet and colleagues (1999) has demonstrated that in addition to the exposure of PS by dying cells, during phagocytosis the phagocyte itself must also expose PS. Thus, if PS bearing aged platelets are phagocytosed by PS bearing endothelial cells, or splenic macrophages, what are the thrombogenic implications of such an event? Speculatively, do patients with thrombocythaemia, who are well characterised to be at high risk from thrombosis, have excess numbers of circulating effete platelets which "overload" the clearance system? Could this increase in clearance demand result in the raised levels of PS exposed by platelet and vasculature, initiating a thrombogenic event? A similar argument may also apply to patients who suffer from sickle cell anaemia, which is characterised by PS exposure on the red cells and thrombosis, particularly infarction of the spleen.

The effectors of the morphological and biochemical changes witnessed during platelet death have yet to be elucidated. Interestingly, the death pathway as we have reported is very reminiscent of a form of cell death known as "aborted apoptosis" whereby a standard apoptosis program is initiated, but is blocked at the level of caspase activation and is finally terminated by alternative caspase-independent routes (Nicotera *et al* 1999). In this respect the increases in proapoptotic Bak and Bax within aged platelets is likely to cause the mitochondrial dysfunctional and release of cytochrome-C detected, but whether this can directly activate non-caspase death effectors, or whether loss of respiratory potential in general can activate factors is unknown. Interestingly, expression of Bax alone in a caspase-inhibited system has been shown to be capable of inducing a form of cell death characterised by loss of mitochondrial potential, cytosolic vacuolation, and increased membrane permeability to Hoechst, but without DNA degradation (Xiang *et al* 1996). However, no mechanism or effectors of these changes have been proposed. Many other non-caspase proteases have been implicated to act in support roles to caspases, or essential cofactors in a wide range of apoptotic systems. These include cathepsins, calpains, serine proteases, granzymes, and the proteosome complex (Leist and Jaattela 2001), with many shown to be able to cleave some of the "classic" caspase substrates. Much work could be directed at investigating the roles of these other proteases in

platelet death (and the apoptosis field in general), by combining genetic approaches with meticulous titration of the pharmacological inhibitors, which are often highly non-specific at concentrations widely used (Johnson 2000).

The novel findings and unanswered questions arising from the MK work alone are numerous. Clearly a fundamental difference must exist between MKs and other cell types given the gross morphological changes witnessed on induction of apoptosis via Fas ligation. As referred to in the introduction and previous discussions, many cytoskeletal components are targeted by caspases in most cell types, so what is the key event or substrate cleaved that makes so much difference in the MK? PKCα has been shown to be required for proplatelet formation (Rojnuckarin and Kaushansky 2001), and has also been demonstrated to be activated in many other cell types during apoptosis (Emoto *et al* 1995; Webb *et al* 2000), none of which extend such projections. On a minimalistic level the early emergence of proplatelet projections, before the platelet-sized nodes form, appear morphologically similar to the multiple processes extended by mature dendritic cells (DCs) (O'Doherty *et al* 1993). Such projections in the DCs have been demonstrated to be dependent on the activation of Rho and Rac (Kobayashi *et al* 2001), members of the Ras-related superfamily of small GTPases well known to be important in membrane rearrangement, filopodia, and lamellipodia formation (Nobes and Hall 1995). A potentially easy pharmacological investigation using inhibitors of this family may prove fruitful, but commercial availability of such reagents is limited, and accessibility to them has tended to be through academic institutions.

A major limitation of much potential *in vitro* work is the relatively low frequency at which either primary or cell line MKs extend proplatelets, even when Fas induced. Hence, any basic biochemical approach using Western blotting will not use a protein lysate that is "all proplatelet" derived. Theoretically a purification strategy able to even partially enrich this delicate phenotype would greatly aid in furthering the work. Initial attempts using velocity sedimentation with BSA gradients to retard the "spindly" branched proplatelet cells have only been of a limited success, with around 15% purity – not enough. A second approach could be to try and subclone the MEG-01 cell line itself. Work in this has demonstrated there to be a wide variation in the level of Fas expression on

the cells, with perhaps only the high expressing cells responding to CH.11 stimulation. Therefore the potential exists to sort these cells either immunomagnetically or with a FACS, followed by expansion of these high level Fas progenitors. Alternatively the cell line could be sorted and cloned for MKs expressing high levels of markers associated with maturity, such as CD42a and/or CD41/61.

The use of *in vivo* models to support and extend this work may prove technically difficult. Although much *in vivo* evidence from genetic ablation of the apoptotic machinery circumstantially supports a role in platelet formation, the major phenotype of thrombocytopenia almost always occurs in association with anti-platelet antibodies, due to defective thymocyte selection (Hashimoto *et al* 2000), and thus the potential for increased immune mediated clearance of platelets. Theoretically primary MKs could be grown *ex vivo* from mice genetically ablated in caspase-3, caspase-9, or caspase-8, thus avoiding any autoimmune issues. However, the homozygotes for caspase-3 and -9 die perinatally and are born at a very low frequency (Kuida *et al* 1996, 1998; Hakem *et al* 1998), the caspase-8 k/o is lethal in utero (Varfolomeev *et al* 1998), and the recovery of haematopoietic colony-forming cells from these embryos is very low. The potential does exist to do a bone marrow adoptive transfer experiment. Bone marrow from, for example, the *gld* mice deficient in active Fas ligand could be transferred into an irradiated control background recipient, whereby the Fas ligand negative cells home to the marrow and repopulate it. Such an experiment may give an insight into whether Fas ligand directed killing is important in constitutive platelet production *in vivo*. Similarly, one could study the rates of platelet recovery between *gld* and control mice following acute platelet loss, such as the administration of anti-platelet antibodies, to examine whether the Fas system is perhaps involved in a more acute platelet recovery response. Unfortunately many possibilities are limited by current animal licence procedures, and the timescale to make amendments to current licences.

This work presented in this thesis not only reveals an important insight into the mechanisms of thrombopoiesis, but also into fundamental evolutionary aspects of cell death in general. The MK/platelet system is both phylogenically and developmentally old, with MKs detectable within the murine yolk sack as early as 7 d postcoitum (Xu *et al* 2001). These rapid maturing MKs have been proposed to give rise to large amounts of platelets, thus preventing haemorrhage in the developing

blood vessels. Interestingly, the major wave of apoptosis during murine development occurs in the brain around 12 d postcoitum (Kuida *et al* 1996, 1998; Hakem *et al* 1998), and therefore the early wave of platelet production may speculatively be one of the earliest apoptotic events during development. The question of how or why did the two ancient processes of thrombopoiesis and apoptosis co-opt presents a fascination idea for contemplation.

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Constitutive Death of Platelets Leading to Scavenger Receptor-mediated Phagocytosis

A CASPASE-INDEPENDENT CELL CLEARANCE PROGRAM*

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Apoptosis is a physiological program for the deletion of cells in which caspases govern events leading to safe clearance by phagocytes. However, a growing weight of evidence now suggests that not all forms of programmed cell death are caspase-dependent. We now report a complete and constitutive but caspase-independent program for the specific phagocytic clearance of intact effete platelets, anucleated blood cells of critical importance in health and disease. Platelets aged *in vitro* not only exhibited increased expression of proapoptotic Bak and Bax but also evidenced constitutive diminution of function such as decreased aggregation to ADP, which was accelerated by culture in the absence of plasma. This abrogation of cell function in plasma-deprived platelets was associated with morphological and biochemical features similar to those of granulocyte apoptosis, that is, cytoplasmic condensation, plasma membrane changes including exposure of phosphatidylserine and the granule protein P-selectin, and recognition by phagocyte scavenger receptors. However, and in contrast with constitutive death of other inflammatory blood cells by apoptosis, these events were not affected by caspase inhibitors, nor was there evidence of caspase-3 activation either by hydrolysis of analog peptide substrates or Western blot analysis, serving to emphasize that neither programmed cell death nor clearance by phagocytes need involve caspases.

Apoptosis has attracted intense scrutiny as a self-contained and physiological program for deletion of unwanted cells (1, 2). Caspase activation has emerged as a key effector mechanism responsible for many of the classical phenomena of apoptosis (3–5) including the first biochemical marker of this type of cell death, endonuclease activation (6, 7). Furthermore, caspases have been persuasively implicated in directing plasma membrane changes that lead to the key physiological outcome of apoptosis, the nonphlogistic recognition and uptake of the intact dying cell by phagocytes (8).

However, the study of caspase-mediated cell death by apoptosis has been dominated by experiments that have frequently relied on artificially induced death in transformed cells and that have rarely paid attention to their recognition and clearance by phagocytes. Nevertheless, these criticisms have been addressed in studies of primary blood cells freshly isolated from healthy human donors (9). For example, neutrophil granulocytes purified by methods designed to minimize artifactual activation (10) undergo constitutive apoptosis that clearly directs specific recognition by phagocytes (11, 12). Furthermore, not only is there evidence that "machinery" caspases such as caspase-3 are activated as neutrophils aged in culture undergo constitutive apoptosis (13–15), but it is also possible to inhibit both apoptosis and recognition by phagocytes using broad spectrum caspase inhibitors such as zVAD-fmk[1] (16, 17). Indeed, in this system, zVAD-fmk inhibits even the very earliest plasma membrane changes that lead to binding of aged neutrophils by phagocytes (17).

The platelet is another blood cell with a short *in vivo* half-life (18). Platelets are critical for normal hemostasis, but disorders of platelet number and function are common, giving rise to a range of bleeding or thrombotic disorders, including stroke and myocardial infarction. Given their importance, it is remarkable that there has been very little study of the constitutive death program that likely accounts for platelet deletion *in vivo*. An important preliminary study (19) suggested that platelets may undergo an apoptotic program because increases in the expression of proapoptotic members of the Bcl-2 family of death-regulating proteins were observed following treatment with ionomycin, a calcium ionophore that induces apoptosis in a range of cell types, most notably lymphocytic cells (20, 21). Interestingly, this study showed that ionomycin-induced increases in Bax and Bak, as well as the cell surface expression of PS, were unaffected by zVAD-fmk and were hence caspase-independent.

To investigate whether platelets exhibited a constitutive death program, freshly isolated platelets were cultured for up to 24 h at 37 °C. In the presence of plasma we observed an increase in levels of proapoptotic Bax and Bak, which, in keeping with an earlier report (19), was consistent with the suggestion that platelets might engage a death program. In support of this, we also observed a constitutive loss of aggregation and spreading functions. By depriving platelets of plasma, a puta-

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[1] The abbreviations used are: zVAD-fmk, carbobenzoxy-Val-Ala-Asp-fluoromethylketone; CM-Orange, ((4-chloromethyl)benzoyl)aminotetramethylrhodamine; FITC, fluorescien isothiocyanate; HBSS, Hanks' balanced salt solution; LDH, lactate dehydrogenase; mAb, monoclonal antibody; Mϕ, monocyte-derived macrophage; PBS, phosphate-buffered saline; PPP, platelet poor plasma; PRP, platelet-rich plasma; TEM, transmission electron microscopy; PS, phosphatidylserine.

tive source of survival factors, not only was loss of function accelerated, but there was also revelation of a cell death program characterized by cytoplasmic condensation, retention of plasma membrane integrity, display at the cell surface of phosphatidylserine and P-selectin, and specific recognition by phagocyte scavenger receptors. However, there was no evidence of caspase-3 activation, emphasizing that constitutive cell death in platelets represents a complete but caspase-independent program leading to phagocyte clearance of intact effete cells.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were of analytical reagent grade and purchased from Sigma unless stated otherwise. Percoll was obtained from Amersham Pharmacia Biotech; sodium citrate solution was from Pharma Hameln GmbH (Hanover, Germany); HBSS without Ca^{2+} and Mg^{2+}, pH 6.4, RPMI 1640, Iscove's modified Dulbecco's medium, and supplements (penicillin, streptomycin, glutamine, and fetal bovine serum) were from Life Technologies, Inc.; FITC and/or phycoerythrin-conjugated mAbs to CD61 (clone BL-E6) and CD62P (clone CRC81) were from Caltag Laboratories (TCS Biologicals Ltd., Botolph Clayton, UK); anti-CD42a (clone GRP-P) was from Serotec Ltd (Kidlington, UK); unconjugated anti-CD62P clone CRC81 and G1–4 were from Caltag Laboratories and Ancell Corporation (Bayport, MN), respectively; FITC-conjugated annexin-V was from BioWhittaker UK Ltd (Wokingham, UK); pan interleukin-1β-converting enzyme (Ab-1, catalog number PC84), caspase-1-pNA substrate (400025), -AFC substrate (688225), caspase-3-pNA substrate (235400), -AMC substrate (235425), acetyl-leucyl-leucylnorleucinal, calpeptin, zVAD-fmk, and Asp-Glu-Val-Asp-fluoromethylketone were from Calbiochem Novachem (Nottingham, UK); anti-human caspase-3 polyclonal antibody (catalog number 65906E) and anti-cytochrome *c* mAbs (clones 6H2.B4 and 7H8.2C12) were from PharMingen (Becton Dickinson, Cowley, UK); PermeaFix was from Orthodiagnostics; and JC-1 (catalog number T3168) and CM-Orange (catalog number C2927) were from Molecular Probes (Leiden, The Netherlands).

Platelet Isolation and Culture—Freshly drawn venous blood was obtained from aspirin-free healthy donors, and PRP was prepared following citration and centrifugation at 300 × *g* for 20 min. PPP was prepared from PRP by centrifugation at 1200 × *g*. Washed platelets were prepared by diluting PRP with 5 volumes of HBSS, pH 6.4, containing EDTA (4 mM) into a round-bottom capped polystyrene centrifuge tube before centrifugation at 280 × *g* for 20 min. Platelets were resuspended and washed in HBSS, pH 6.4, before finally resuspending in either PPP, Iscove's Dulbecco's modified Eagle's medium, or HBSS, pH 6.4. Platelets were maintained at 37 °C in a closed (capped) tube at 3×10^8/ml.

Following isolation, for those experiments involving phagocytic recognition of aged platelets, platelets were first incubated in HBSS at 5×10^9/ml for 10 min with 1.8 μM CM-Orange. Unincorporated dye was removed with two washes of HBSS, pH 6.4, before resuspending at 5×10^8/ml in HBSS, pH 6.4. Platelet preparations were routinely checked by microscopy for the presence of aggregates, which if found resulted in the experiment being discarded.

Platelet Aggregation Experiments—Platelet aggregation studies were performed in a PAP4 aggregometer (Bio-Data Corporation) in which 0.5-ml aliquots of platelets, resuspended in PPP, were incubated for 2 min while stirring at 37 °C before the direct addition of agonist. Prior to aggregation experiments, washed platelets were harvested by centrifugation at 280 × *g* for 20 min and resuspended in autologous PPP. Agonists used were ADP (10 μM), thrombin (10 units/ml), platelet-activating factor (10 μM), and U46619 (10 μM). In some instances, platelets were preincubated for 1 min with the anti-aggregating reagent MK852 (10 μM) prior to the addition of agonist.

Platelet Adhesion and Cell Spreading on Collagen—Glass microscope slides were coated with either collagens I or IV (100 mg/ml solutions in PBS, pH 7.4). After 30 min the slides were washed exhaustively with PBS before applying a volume of suspended platelets in cultured media. Following a 20-min incubation, the slides were flicked free of media and nonadherent cells and snap-frozen with methanol and acetone (1:1). The slides were then air-dried before staining with phalloidin-FITC in PBS (2 μg/ml). Slides were examined by epifluorescent microscopy (Nikon Diaphot 300).

Sample Preparation for Transmission Electron Microscopy—Cell suspensions were prepared for electron microscopy by first washing the platelets with PBS and fixing with 2.5% (v/v) glutaraldehyde in PBS for at least 1 h at 4 °C. Cells were then pelleted at 280 × *g* before resuspending in a sodium cacodylate buffer, pH 7.4, containing 2.5% glutaraldehyde and leaving overnight. Samples were recentrifuged at 280 × *g*, the supernatant was removed, and the cells were overlaid with a minimal volume of PRP-derived serum, which in turn was overlaid with the cacodylate buffer for at least 1 h at room temperature. The pellet was then transferred to fresh fixative and treated as normal resected tissue for TEM.

Monocyte Isolation and Culture—Human monocytes were isolated from freshly drawn venous blood following citration, dextran sedimentation, and plasma-Percoll density gradient centrifugation as described previously (10, 11). Human monocyte-derived macrophages (Mϕ) were obtained by the standard technique of culturing adherent monocytes for 5–7 days in Iscove's Dulbecco's modified Eagle's medium plus 10% PRP-derived serum (11, 22).

Phagocytic Recognition of Aged Platelets—Platelets labeled with CM-Orange and aged in culture were washed free of conditioned media and resuspended in HBSS before addition to a prewashed monolayer of adherent phagocyte cell lines cultured in 24-well plates. Typically 5×10^7 platelets were incubated with 1×10^5 phagocytes at 37 °C for 10 min for platelets aged in the absence of plasma and 30 min for platelets aged in citrated plasma. Following the incubation period, the phagocyte monolayer was washed free of noninteracting platelets, and any adherent platelets were removed by treatment with trypsin at 37 °C for 5 min followed by 5 mM EDTA at 4 °C, to recover the human Mϕ, and trypsin/EDTA treatment at 37 °C for 15 min for all other cell lines tested, before flow cytometric and epifluorescent microscopic analysis.

Immunolabeling and Fluorescence-activated Cell Sorter Analysis of Platelets—Immunofluorescent labeling of intact platelets for surface expression of CD42a, CD61, or CD62P was typically performed by resuspending 5 μl of cultured platelets with 40 μl of the appropriate FITC-conjugated mAb (diluted 1:1000 with 10% new born calf serum in PBS) for 10 min before adding 400 μl of fluorescence-activated cell sorter sheath fluid and sampling by flow cytometry using a single laser FACScan (Becton-Dickinson, Mountain View, CA).

Intracellular immunolabeling of the Bcl family of proteins was performed following fixation and permeabilization of the platelets with PermeaFix (1 ml/5×10^8 platelets for 30 min on ice). Excess fixative was then removed with two washes of PBS before resuspending with 10% new born calf serum in PBS at 5×10^8/ml. 5×10^6 platelets were then incubated overnight with neat antibodies to Bak (1 μl), Bax (1 μl), Bcl-2 (1 μl), Bcl-x (1 μl), and Mcl-1 (1 μl) or their appropriate negative controls. All primary antibodies were detected with FITC-conjugated $F(ab')_2$ fragments of sheep anti-rabbit polyclonal antibodies or goat anti-mouse polyclonal antibodies from Sigma.

Phosphatidylserine exposure by intact platelets was determined by resuspending 5 μl of cultured platelets in 400 μl of HBSS containing 10 mM Ca^{2+} and 10 μg/ml of FITC-conjugated annexin-V. All labeling steps were maintained at 4 °C.

SDS-Polyacrylamide Gel Electrophoresis and Protein Blotting—For detection of caspase-3, platelets were lysed by resuspending in SDS Laemmli sample buffer and immediately boiled for 5 min. For cytochrome *c* detection, washed platelets were resuspended in ice-cold 10 mM Hepes buffer (containing 1 mM EDTA, 1 mM 1,10-phenanthroline, 1 mM phenylmethylsulphonylfluoride, 1 mM benzamidine, 10 μM pepstatin, 10 μM leupeptin, 10 μM antipain, pH 8.0) and lysed following two rounds of freeze-thaw. The lysed platelets were then centrifuged at 13,000 × *g* to yield a cytosolic supernatant (S13), and the pellet was resuspended in lysis buffer before centrifuging again and dissolving the pellet in 1% TX-100 and recentrifuging at 13,000 × *g* to yield soluble protein from intact mitochondria (T13). The S13 and T13 fractions were then precleared with a control IgG and protein G-agarose before immunoprecipitating cytochrome *c* with clone 6H2.B4 and boiling in SDS sample buffer (Laemmli). Protein was analyzed on the basis of equal numbers of extracted cells rather than on the amount of protein loaded. Immunodetection of transblotted protein to polyvinylidene difluoride membranes was performed as described previously (13).

Spectrophotometric Assay for Lactate Dehydrogenase—Aged washed platelets were incubated overnight and pelleted by centrifugation at 280 × *g*. The supernatant was removed, and the pellet was resuspended to the same volume in HBSS and sonicated. Stock solutions of NADH (0.2 mM) and sodium pyruvate (1.6 mM) were freshly prepared in a Tris (81.3 mM)/NaCl (203.3 mM) buffer, pH 7.2. The assay was initiated by the addition of platelet supernatant or sonicate (40 μl) to a quartz silica cuvette maintained at 37 °C and containing 420 μl of NADH and 80 μl of pyruvate. LDH activity was measured as the time-dependent and pyruvate-dependent decrease in the absorbance of NADH at 339 nm. LDH activity was expressed as μmoles of NADH consumed per min per ml of platelet supernatant or sonicate.

RESULTS

Cultured Platelets Exhibit Increased Levels of Proapoptotic Bak and Bax—An important determinant of whether a cell will undergo apoptosis is its intracellular balance between anti-apoptotic members of the Bcl-2 protein family such as Bcl-2 and Mcl-1 and proapoptotic members such as Bax and Bak (23, 24). The possibility that platelets might be able to undergo an apoptosis-like death has been raised by Vanags *et al.* (19) who reported that ionomycin, which triggers apoptosis in many cell types (20, 21, 25), caused an increase in the expression of proapoptotic Bax and Bak, but not Bcl-2. To extend the findings of Vanags *et al.* we decided to study a different model system in which apoptosis-like death might occur. Granulocytes "aged" in culture undergo constitutive death that is accelerated in the absence of survival factors (26–28) and is correlated both with increased levels of Bax (29, 30), but not Bak (31), and decreased levels of Mcl-1 (32). We therefore tested the hypothesis that prolonged culture of platelets might also lead to an increase in the expression of proapoptotic Bcl-2 family members and a susceptibility to apoptosis-like death.

By immunofluorescence flow cytometry we confirmed that freshly isolated platelets expressed Bax, Bak, and Mcl-1, but not Bcl-2 (Fig. 1). The ability of each antibody to recognize its antigen was verified both by flow cytometry and Western blot analysis with appropriate control cell lines.[2] Interestingly, the levels of immunodetectable Bak and Bax increased by 3.4- and 2.4-fold, respectively, as platelets were aged for 18 h in citrated plasma (Fig. 1, *B* and *C*). No significant changes in Mcl-1 were apparent. In keeping with studies of ionomycin stimulation of platelets (19), these data suggest that platelets cultured for 18 h can adopt a more proapoptotic balance and further suggest that aging in culture, as originally reported for neutrophils (11), was likely to be a useful model for platelet cell death. Furthermore, a tendency to apoptosis-like death during culture was reinforced by the observations that after 18 h of culture, aged platelets exhibited diminished mitochondrial membrane potential as measured by JC-1 and release of cytochrome *c* into the cytoplasm (data not presented).

Because plasma is a potential source of exogenous survival factors, we went on to seek evidence that plasma deprivation, which resulted in increased levels of Bax and Bak comparable with those observed for platelets aged in the presence of plasma (data not shown), might accelerate and therefore reveal a constitutive death program overlooked in previous short term culture experiments on platelets.

Aged Platelets Exhibit Impaired Function—Platelets possess many of the functional responses exhibited by other inflammatory blood cells and a key feature of apoptosis in leukocytes that are cultured overnight in the presence of serum is loss of the ability to respond to external stimuli and mount pro-inflammatory responses (33). Interestingly, abrogation of cell function is characteristic of platelets that have been stored at 37 °C in the presence of plasma, especially the inability to respond to weak agonists such as ADP. With the use of an aggregometer, which measures the light transmittance of a stirred platelet suspension, we were able to confirm that platelets lost the ability to aggregate but not to undergo a shape change in response to ADP when cultured overnight in the presence of citrated plasma (Fig. 2).

Treatment of freshly isolated (viable) platelets with 10 μM ADP resulted in an immediate shape change, observed as a slight decrease in light transmittance (upward deflection in Fig. 2*A*), followed by an irreversible decrease in light transmittance that was indicative of a full aggregation response (Fig. 2*A*). By reducing the concentration of ADP to 3 μM and in accordance with the biphasic nature of platelet aggregation (34), we observed a reversal of the initial wave of aggregation because of the absence of endogenous agonists such as ADP being secreted by the weakly activated platelets (Fig. 2*A*). In

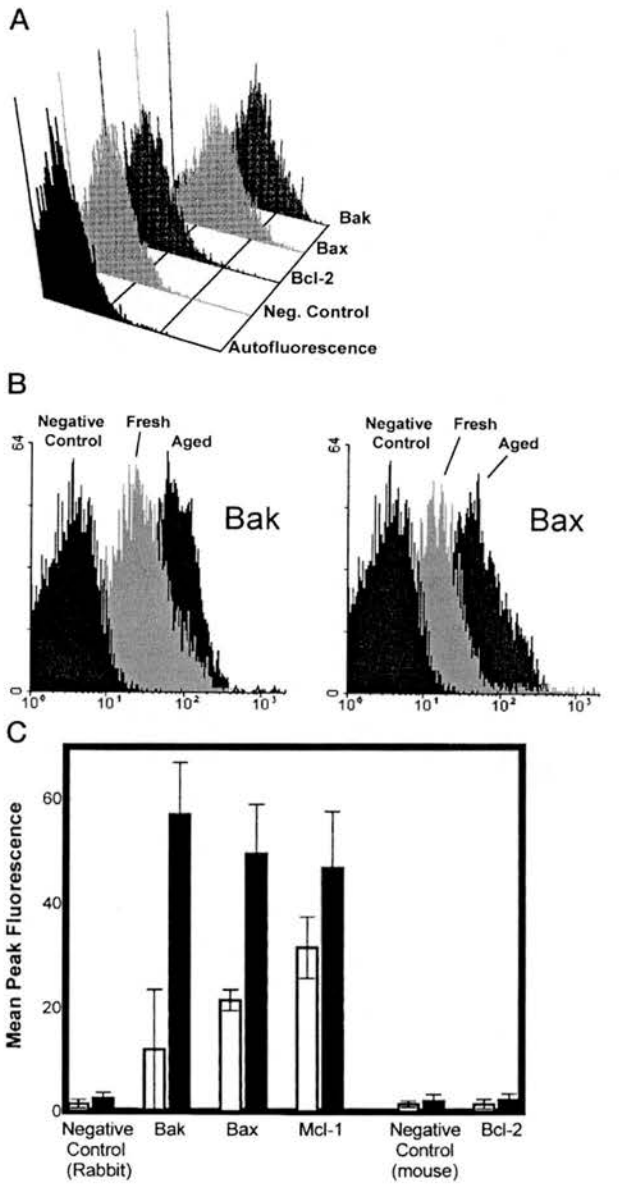

FIG. 1. **The proapoptotic balance of Bcl homologs is accentuated in aged platelets.** *A*, typical immunofluorecence flow cytometry histograms of fixed permeabilized (PermeaFix) cells that demonstrate that freshly isolated platelets express the proapoptotic Bcl homologs Bak and Bax but not the anti-apoptotic homolog Bcl-2. The negative controls for mouse (Bcl-2) and rabbit (Bak, Bax, and Mcl-1) antibodies were superimposable and no different from autofluorescent controls, confirming an absence of nonspecific binding. *B*, overlays of representative histograms showing the shift in fluorescence for Bak and Bax expression between freshly isolated and aged platelets. Autofluorescence and control irrelevant staining for fresh and aged platelets were essentially indistinguishable. *C*, the mean peak fluorescence for various members of the Bcl family is presented as the arithmetic mean ± 95% confidence interval for $n = 4$ separate experiments (three different donors). Each experiment was performed in duplicate on samples permeabilized with PermeaFix with fresh citrated platelets (*open columns*) or platelets cultured in the presence of citrated plasma for 18 h (*solid columns*).

[2] L. Magowan, unpublished observations.

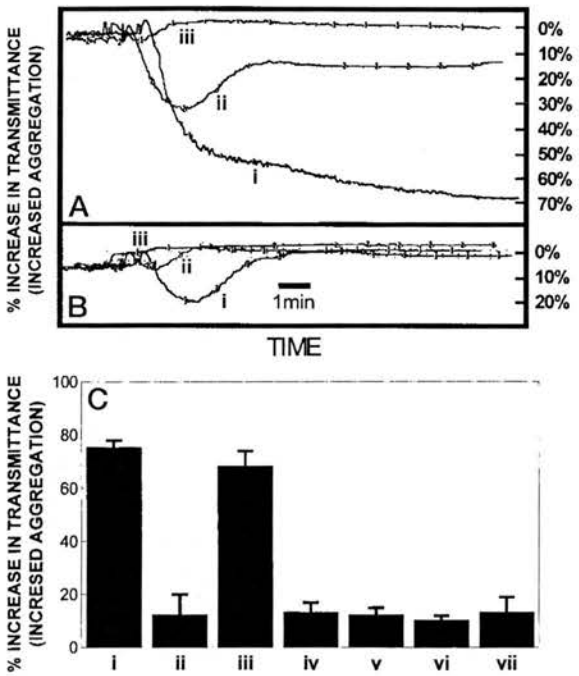

FIG. 2. **Agonist-induced aggregation is down-regulated in aged platelets.** Photometric measurement of stirred suspensions of freshly isolated platelets (*A*) or platelets aged in citrated plasma for 24 h (*B*) were monitored for changes in light transmittance over a 10-min period following activation with ADP to a final concentration of 10 μM (*i*), 3 μM (*ii*), and 1 μM (*iii*). The upward deflection (decreased transmittance) in the traces immediately following the addition of ADP is indicative of shape change, whereas increases in transmittance are indicative of reversible (*ii*) or full aggregation (*i*). The chart recorder moved from *left* to *right* with a 1-min interval represented by the *horizontal bar* in *B*. *C*, freshly isolated platelets were maintained for 4 h at 37 °C in either citrated plasma (*i*) or, after being washed free of plasma, in HBSS (*ii*) containing either thrombopoietin (5 ng/ml) (*iv*), platelet-derived growth factor (10 ng/ml) (*v*), insulin-like growth factor-1 (100 ng/ml) (*vi*), or bovine serum albumin (4 mg/ml) (*vii*). Alternatively, washed platelets were returned to PPP (*iii*). ADP was then added to a final concentration of 10 μM, and the percentage of change in light transmittance was recorded for 10 min. *Error bars* represent the 95% confidence interval for five separate experiments.

the presence of 1 μM ADP there was no evidence of aggregation, although shape change, which is the most sensitive response of platelets, persisted. Aggregation, but not shape change, could also be blocked by preincubating freshly isolated platelets with the anti-GPIIb reagent MK852 (data not presented). In comparison, platelets aged over a 24-h period in plasma lost the ability to aggregate ($t_{1/2}$ = 12 ± 2 h) but not to undergo a shape change in response to ADP (Fig. 2*B*). The inability of aged platelets to respond was also observed with the agonist platelet-activating factor and the endoperoxide analog U46619 (data not presented).

In keeping with the hypothesis that plasma might contain survival factors that normally retard constitutive platelet death, we found that the loss of an aggregation response to ADP was markedly accelerated when platelets were washed and cultured in the absence of plasma ($t_{1/2}$ = 1.5 ± 0.5 h). This rapid loss in ADP-induced aggregation by washed platelets was prevented and returned to rates comparable with those of unwashed platelets maintained in plasma by reconstituting the washed platelets with PPP ($t_{1/2}$ = 12 ± 2 h) (Fig. 2*C*). This indicated that the loss in platelet response was not dependent on the washing procedure *per se* but rather on the absence of permissive factors within plasma. Culturing washed platelets in the presence of the megakaryocyte differentiation factor thrombopoietin and known survival factors in other cell systems such as platelet-derived growth factor, granulocyte-macrophage colony-stimulating factor, and insulin-like growth factor-1, had no effect. As a control for oncotic effects we tested bovine serum albumin at 4 mg/ml and found no protective effect and could eliminate glucose as a confounding factor given that its concentration in HBSS (1 mg/ml) is equivalent to plasma concentrations (0.7–1.1 mg/ml). Initial investigations to characterize the putative soluble plasma survival factor(s) by dialysis have revealed the activity to be of 50 kDa or greater in molecular size and stable to long term storage at 4 °C and −20 °C.

Constitutive loss of platelet function on incubation at 37 °C was further confirmed by assessing the ability of aged platelets to adhere and spread on collagen coated surfaces with the formation of lammelipodia and filopodia. Microscopic examination of phalloidin-FITC-stained preparations revealed that freshly isolated platelets readily adhered to both collagen I- and IV-coated glass slides, whereas aged platelets, whether cultured in the presence or absence of plasma protein, did not (data not presented).

Aged Platelets Maintain Plasma Membrane Integrity—Down-regulation of cell function is a feature of programmed cell death in other blood cells (33, 35, 36) but might also have reflected necrosis. Assessing necrosis was not straightforward in platelets because their small size precluded the use of vital dyes such as Trypan Blue because admission of dye could not be confidently assessed by light microscopy. Similarly, the absence of a nucleus precluded the use of DNA staining vital dyes such as Hoechst and propidium iodide. Nevertheless, flow cytometry revealed that the forward and side scatter properties of fresh and aged platelets, whether cultured in HBSS or PPP, were virtually superimposable (data not presented). This contrasted with the deliberate impairment of plasma membrane integrity by hypotonic lysis, thermal treatment, or mild acid treatment that invariably resulted in the appearance of cellular debris and the loss of platelets as assessed by forward and side scatter (data not shown).

Further evidence against necrosis being a confounding factor in the constitutive loss of platelet function was the use of phalloidin-FITC as an actin-binding "vital dye" where greater than 99% of both fresh and aged platelets excluded the dye when assessed by flow cytometry (Fig. 3*A*). As a positive control to reveal the potential level of intracellular staining, aged platelets were permeabilized with the fixative PermeaFix prior to staining with phalloidin-FITC. Fluorescence microscopy confirmed that phalloidin-FITC had stained intracellular F-actin of permeabilized platelets (data not presented). These data were a strong indication that aged platelets were capable of maintaining plasma integrity under the conditions employed in this study.

As further confirmation of platelet integrity, we assessed the level of LDH activity, an abundant intracellular enzyme of platelets, in the supernatants of cultured platelets (Fig. 3*B*). Importantly, less than 4% of total LDH activity was found in the supernatant of platelets aged for 18 h in HBSS in the absence of serum. To confirm that soluble LDH maintained under comparable conditions was stable, we cultured whole cell lysates from fresh platelets either on its own or with erythrocytes. These experiments revealed that soluble LDH was stable in culture over a 24-h period (Fig. 3*B*). The 75% loss in LDH activity in the cytosol of intact aged platelets appeared likely to reflect intracellular catabolism of retained LDH or its inactivation by, for example, transglutaminases. Unfortunately, given the high levels of LDH in plasma, we were unable to reliably assess the release of LDH from platelets aged in

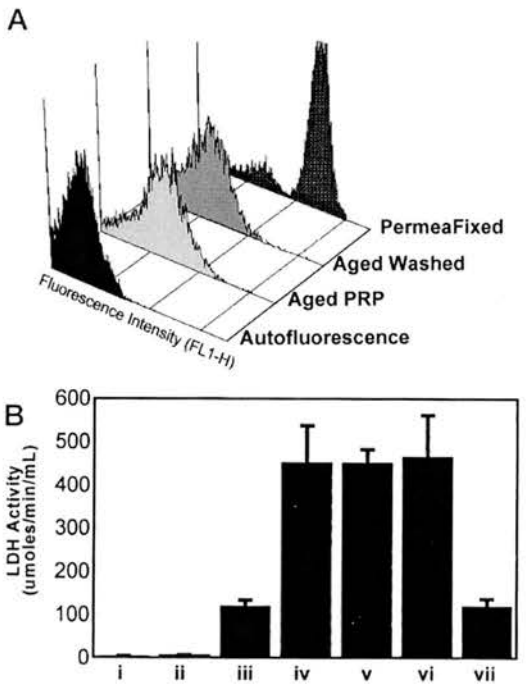

FIG. 3. **Aged platelets maintain plasma membrane integrity.** *A*, flow cytofluorimetric analysis for platelet membrane integrity of fresh and aged platelets using actin-binding phalloidin-FITC as a vital dye. Suspensions of platelets aged in the presence (*Aged PRP*) or absence of plasma (*Aged Washed*) were washed free of conditioned media and incubated for 10 min on ice with phalloidin-FITC at 2 μg/ml in PBS before flow analysis. The FL1 channel (FITC) on a Becton-Dickinson FACScan was set to the autofluorescence of unlabeled freshly isolated washed platelets. As a positive control for phalloidin-FITC staining, aged platelets cultured in the absence of serum were permeabilized with PermeaFix (see "Experimental Procedures"). *B*, freshly isolated platelets were resuspended in HBSS and cultured in the absence of plasma for either 1 or 18 h before separating the cells from the conditioned supernatant and resuspending the cells in an equivalent volume of fresh HBSS and sonicating. Clarified supernatants of fresh (*i*) and aged (*ii*) washed platelets or their cell lysates (*iii* and *iv*, respectively) were monitored for LDH activity. Cell lysates from fresh platelets were also incubated for 18 h in HBSS either on their own (*v*) or in the presence of erythrocytes (*vi*). The level of LDH in PPP is shown for comparison (*vii*). *Error bars* represent the 95% confidence interval of four separate experiments each done in duplicate.

plasma. Taken together with the phalloidin data, these results provide strong evidence against the possibility that aged platelets may have undergone necrosis but rather may have undergone a form of programmed cell death.

Transmission Electron Microscopy of Aged Platelets Is Suggestive of a Cell Death Program—To further eliminate the possibility of necrosis we prepared samples for TEM. Freshly isolated platelets, whether washed or not, exhibited characteristic discoid-like features of nonactivated platelets (Fig. 4). This was confirmed by the absence of filopodia and cell surface protrusions or a centrally clumped body of organelles surrounded by a circumferential band of a constricting microtubular network. A cross-section through the platelets also revealed the typical distribution of dense bodies, α-granules, mitochondria, and glycogen particles.

However, platelets aged in culture under conditions leading to loss of function exhibited dramatic morphological changes. Control platelets maintained in citrated plasma for 24 h exhibited few changes from the freshly isolated state, except that the majority of cells assumed a spherical rather than a discoid shape. However, when aged for 12 h in HBSS in the absence of serum, when loss of function was complete, there was remarkable condensation of cytoplasm and granules with submembrane vacuolization reminiscent of that reported during apoptosis in megakaryocytes (37). Interestingly, cultured platelets did not exhibit plasma membrane blebbing that is a common feature of many cell types undergoing apoptosis, but lack of surface blebbing is a notable feature of granulocyte apoptosis (11). Furthermore, in keeping with evidence of granule fusion with the plasma membrane as aging neutrophil granulocytes progress to an intact late apoptotic state prior to secondary necrosis (38), platelets aged for 24 h exhibited fusion of granules with the plasma membrane. In view of these morphological changes suggestive of an apoptosis-like program of platelet death with similarities to that observed in granulocytes, we went on to seek comparable plasma membrane changes.

Aged Platelets Exhibit Cell Surface Changes of Apoptosis—Although exposure of phosphatidylserine (PS) in the outer membrane of platelets was originally demonstrated to be a marker of platelet activation with procoagulant properties (39), PS exposure is also recognized as a reliable marker of cells undergoing caspase-dependent cell death (40–42). In granulocytes, PS exposure and caspase activation are tightly linked to nuclear changes typical of apoptosis (14, 16, 17). We therefore sought to determine by flow cytometry the level of PS exposure using FITC-conjugated annexin-V, a high affinity probe for PS (43). Flow cytometric analysis of control platelets aged for 24 h in citrated plasma and labeled with annexin-V-FITC revealed a bimodal distribution with typically no more than 8% found positive (Fig. 5*A*). Although a background level of 0.2–0.5% of fresh platelets bound annexin-V, any increases were not apparent until at least 8 h of culture where annexin-V binding increased steadily to 8% by 24 h with minimal increases seen thereafter. In contrast, washed platelets aged in the absence of plasma, which again contained few annexin-V binding cells in the first 6 h of culture, rapidly switched after 8 h to be >80% positive by 18 h (Fig. 5*A*). To address the confounding possibility that PS exposure was simply a result of the cell having insufficient energy to maintain the "flippase" activity, washed platelets were aged in the presence of varying concentrations of glucose (1 mg/ml to 10 mg/ml). Following assessment of the level of PS exposure by Annexin-V binding and flow cytometry, no significant differences were seen (data not presented). The inability of aged platelets to maintain an asymmetric distribution for PS was strong evidence of an apoptosis-like constitutive cell death program because activated platelets expressing PS possess a translocase that re-establishes an asymmetric distribution, unless platelets have undergone secondary events of aggregation (44).

Furthermore, we sought to confirm morphological evidence of granule fusion with the plasma membrane during constitutive platelet death seen by TEM (Fig. 4) because this is an important cell surface feature of later stages of constitutive apoptosis in neutrophils (38). To assess for this possibility we probed for the intracellular α and dense granule marker P-selectin (45) (Fig. 5*B*). Reassuringly we found that platelets maintained in citrated plasma did not express any cell surface P-selectin in the first 8 h of culture, evidence against platelet activation. Indeed, after aging for 24 h in the presence of plasma we found that only a small proportion (~10%) of platelets expressed P-selectin. However, washed platelets cultured in HBSS in the absence of serum rapidly mobilized intracellular stores of P-selectin following 6 h of *in vitro* culture so that all cells were positive by 7–10 h.

Constitutive Platelet Death Is Caspase-independent—Activation of caspases is reported to be upstream of plasma membrane changes associated with apoptosis including PS exposure (8, 16, 17, 40, 42). Although Western blot analysis confirmed

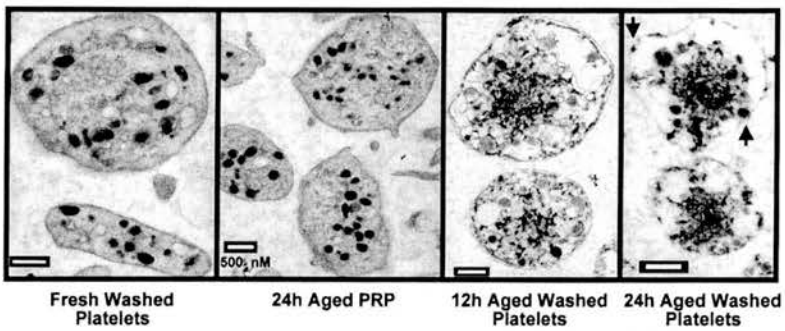

FIG. 4. **Aged platelets exhibit cytoplasmic condensation.** Transmission electron micrographs of platelet preparations are shown: washed platelets fixed immediately following isolation; aged platelets maintained in citrated plasma for 24 h; and washed platelets maintained in HBSS for 12 and 24 h, respectively. Note evidence of granule fusion with the plasma membrane (*arrows*). The *white scale bar* in each panel represents 500 nm.

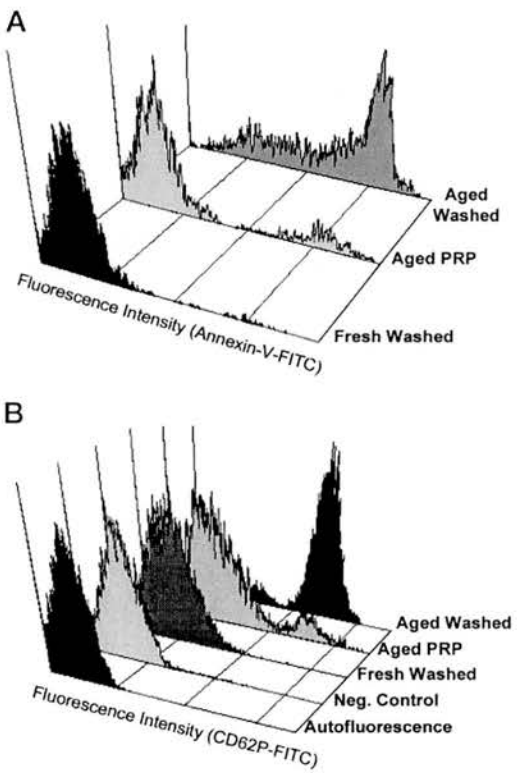

FIG. 5. **Aged platelets express phosphatidylserine and P-selectin at their surface.** Fresh washed platelets, platelets aged in HBSS in the absence of serum protein (aged washed), and platelets aged in citrated plasma (aged PRP) were assessed by flow cytometry for phosphatidylserine exposure using annexin-V-FITC (*A*) and granule cell fusion with the plasma membrane by monitoring for cell surface P-selectin expression using a FITC-conjugated anti-P selectin mAb CRC81 (*B*). The FL1-H channel (FITC) was set to the autofluorescence of unlabeled platelets and confirmed that nonspecific binding of control antibodies was minimal.

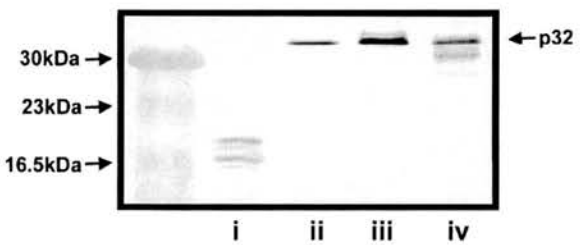

FIG. 6. **Caspase-3 is not cleaved as platelets age in culture.** Cytosolic extracts taken from fresh (*iii*) and aged platelets (*iv*) cultured in the absence of serum were probed for caspase-3 by Western blot with a polyclonal antibody that recognized both the 32-kDa (*p32*) precursor and the17-kDa subunit of the activated enzyme (note lack of the latter). As positive controls, cell lysates were prepared from untreated Jurkat T-cells (*ii*) that exhibited low levels (10%) of constitutive apoptosis as judged by Giemsa stained cytospins, and those induced to undergo apoptosis (*i*) following a 5-h treatment with staurosporine (2 μM) (approximately 65% apoptosis). Positions for the 30-, 23-, and 16.5-kDa mass markers were determined with the use of Rainbow Markers (Sigma).

that caspase-3 was present as a 32-kDa species in platelets that was reduced in aged preparations (Fig. 6), we were unable to identify caspase-mediated cleavage to an active fragment (17 kDa) as was observed with apoptotic Jurkat T cells. Interestingly, aged platelets did reveal the presence of proteolyzed species of molecular weights just less than the p32 parent band, which a very recent report (46) indicates is due to calpain-mediated processing to nonactivated species. In keeping with these and other results (19, 46), and in contrast to apoptotic Jurkat T cells, we were also unable to detect caspase activity using fluorogenic-AFC or chromogenic-pNA substrates for either caspase-1 or caspase-3 (data not presented).

In further agreement with these data we also found that a number of protease inhibitors, including the caspase inhibitors Asp-Glu-Val-Asp-fluoromethylketone and zVAD-fmk, had no effect on the rate of PS exposure (Table I). Additionally, we also found that the inhibitors had no effect on the refractiveness of aged platelets to ADP-induced aggregation (data not presented) whether cultured either in the presence or absence of plasma. Similarly, phagocyte recognition of washed platelets aged in the presence of caspase inhibitors was not different from control, confirming the caspase-independent nature of phagocyte clearance of aged platelets (Table I). Combined, these results suggest that caspases do not have a major role in the constitutive cell death of platelets and complement recent studies on apoptosis-like events associated with platelet activation (19, 46).

Aged Platelets Are Ingested by Phagocytes via Scavenger Receptors—In vivo, the most important feature of the apoptotic cell death program is that intact effete cells are recognized and rapidly ingested by professional and semi-professional phagocytes. In keeping with this, we found that platelets cultured for 18 h in the absence of plasma were readily ingested by 6-day-old human Mϕ following a 30-min phagocytosis assay as evidenced by TEM (Fig. 7).

To quantitate platelet ingestion by a range of phagocytes, we developed a flow cytometric method (Fig. 8) that was dependent on incubating the phagocytes with platelets that had been prelabeled with an orange fluorescing reagent (CM-Orange) (Fig. 8*A*). Phagocytes were then sorted by flow cytometry where

TABLE I
Effect of protease inhibitors on PS exposure and recognition of aged washed platelets

Condition/inhibitor	Concentration	PS-positive washed platelets (after 18 h)	n^a	Recognition of aged washed platelets (normalized)	n
	μM	%		%	
Aged washed		71.2	7	100.0	4
Me_2SO control	[0.1%]	70.6 ± 3.8	7	99.2 ± 4.7	4
zVAD-fmk	100	84.2 ± 8.0	5	117.4 ± 17.0	4
DEVD-fmk	10	76.0 ± 3.5	5	99.5 ± 7.9	4
Acetyl-leucine-leucylnorleucinal	100	71.8 ± 5.9	7	ND[b]	
Calpeptin	100	77.3 ± 9.9	5	ND	

[a] n, number of separate experiments.
[b] ND, not done.

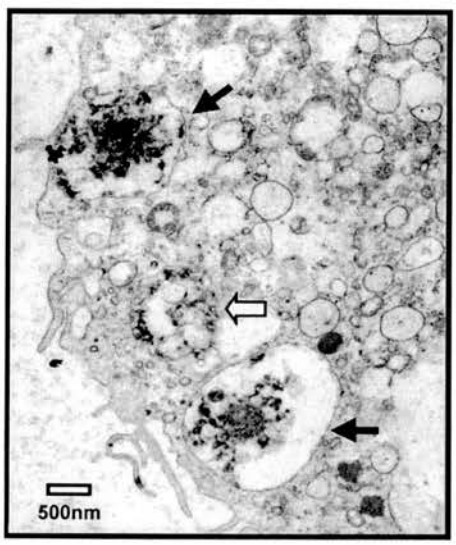

FIG. 7. **Human monocyte-derived macrophages readily ingest aged platelets.** A representative TEM showing a cytoplasmic region just beneath the plasma membrane of a human macrophage that contains distinct evidence of having ingested two (*solid arrow*) and possibly three (*open arrow*) platelets. The *white scale bar* represents 500 nm.

they were readily resolved from platelets by forward and side scatter (Fig. 8*B*) with any shift in orange fluorescence (FL2) attributed to interacting platelets (Fig. 8*C*). To discriminate between adherence and ingestion we also labeled the phagocytes prior to flow cytometry with an FITC-conjugated anti-CD61 mAb. CD61, also known as glycoprotein IIIa, is a specific cell surface marker for platelets (Fig. 8*A*) in which its expression was not found to alter as platelets were aged in culture whether in the presence or absence of plasma. Interestingly, we observed that anti-CD61 mAb routinely failed to label our phagocyte populations, suggesting that platelets were ingested, in keeping with TEM (Fig. 7) and that any adherent platelets were removed prior to flow cytometry. Assessment of cytospin preparations by confocal microscopy also confirmed that platelets were contained within phagocytes (data not presented). In contrast and as a control comparison, we confirmed that freshly isolated platelets adhere to the surface of freshly isolated monocytes (47) (Fig. 8*D*).

Although fresh platelets on microscopic examination were observed to adhere to all phagocytic cell lines tested, only aged platelets were found to be ingested following flow cytometric analysis (Table II). We also observed that the degree of phagocytosis was always greater for platelets aged in the absence of plasma survival factors than those aged in citrated plasma. Typically, after a 30-min interaction, greater than 90% of human Mϕ and Bowes melanoma cells ingested platelets aged in the absence of plasma compared with only 30 ± 12% and 20 ± 6%, respectively, when aged in the presence of citrated plasma. Moreover, time course experiments repeatedly showed that in a fixed time phagocytosis assay the maximal level of ingestion by human Mϕ and Bowes melanoma cells was achieved with platelets aged for 12 h in the absence of plasma or 24 h for those cultured in citrated plasma.

The degree to which cell lines ingested aged platelets, whether cultured in the presence or absence of plasma, was found to be unaffected by various well characterized inhibitors of recognition (Table II) (48). These included the integrin inhibitor RGDS, the PS receptor-competitor phospho-L-serine, the cationic sugars glucosamine and galactosamine, the anti-CD36 mAb SMϕ, and the anti-CD14 mAb 61D3 (Table II). Conclusive evidence against a role for CD36 in phagocytosis was confirmed with the use of Bowes melanoma cells stably transfected with CD36, which exhibited no increase in phagocytosis when compared with control Bowes melanoma cell lines (49). However, greater than 75% inhibition was observed with fucoidan, a recognized inhibitor of the scavenger receptor pathway, in contrast to the lack of inhibition by dextran at the same concentration, which served as control. Polyinositol, another inhibitor of the scavenger receptor pathway, but not its standard control polycytidine also inhibited recognition, although not as effectively as fucoidan (Table II). Nevertheless, further confirmation of a major role for the scavenger receptor was obtained with the anti-murine scavenger receptor mAb 2F8, which inhibited mouse peritoneal macrophage uptake of aged platelets.

Because our studies had also shown that aged platelets expressed P-selectin (Fig. 5*B*), which mediates adhesion of activated platelets to monocytes (50), and given that fucoidan is known to bind the lectin domain of P-selectin, we explored the role of P-selectin in platelet recognition. By using a function-blocking mAb (clone G1) to P-selectin, which recognizes the lectin domain, we found that the recognition of aged platelets by human Mϕ and Bowes melanoma cells was only weakly affected, and no synergy with polyinositol was observed (Table II). This suggests that although P-selectin had a minor role in the phagocytosis of aged platelets it was not primarily responsible for mediating phagocytic recognition and clearance, a conclusion in agreement with others (51).

DISCUSSION

Platelets play a crucial role in hemostasis and thrombosis, with the consequence that they are of central importance in common disorders such as myocardial infarction and stroke. However, little is known of candidate mechanisms for safe clearance of these anucleated blood elements. Prompted by earlier work on constitutive apoptosis in other key blood cells, granulocytes, we sought evidence for a constitutive death program available to platelets. In keeping with earlier work (19) we confirmed that platelets expressed members of the Bcl-2 family of cell death-regulating proteins and observed that there was an apparent proapoptotic shift in platelets aged for 18 h in citrated plasma. We also observed that aged platelets lost the

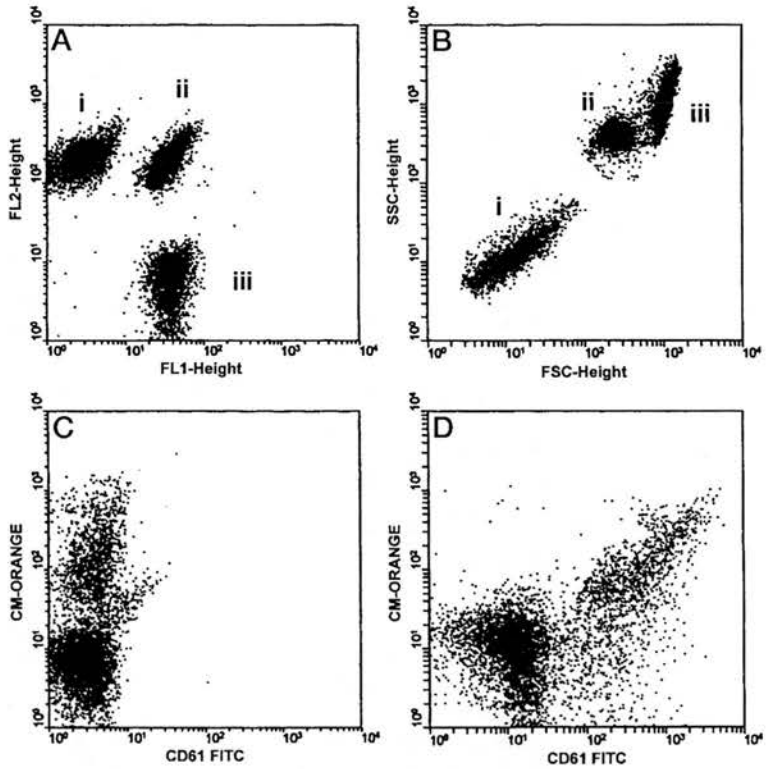

FIG. 8. **Ingestion of aged platelets is readily quantitated with the development of a flow cytometric method.** *A*, human platelets prelabeled with CM-Orange (*i*) and aged in HBSS in the absence of plasma were confirmed to express the platelet specific marker CD61 with an FITC-conjugated mAb (*ii*) whose expression remained unaltered relative to freshly isolated CM-Orange unlabeled platelets (*iii*). *B*, CM-Orange labeled platelets (*i*), incubated with human macrophages (*ii*), or with Bowes melanoma cells (iii) for times indicated elsewhere were readily resolved from the phagocytes according to their forward and side scatter properties in flow cytometry. *C*, CM-Orange-labeled platelets that associated with the phagocyte population are seen as a distinct subpopulation that is shifted in orange fluorescence (*y* axis). These platelets were predominantly ingested given that they failed to dual label with the FITC-conjugated mAb to CD61. Similar results were found with mAbs to other platelet markers such as CD42a. *D*, in contrast, freshly isolated monocytes readily bind but do not ingest freshly isolated CM-Orange-labeled platelets.

TABLE II

Platelet ingestion is mediated by the scavenger receptor

The level of inhibition by the following compounds on the ingestion of platelets that had been aged in either the absence or presence of plasma protein were assessed by flow cytometry as follows: −, 0–5% inhibition; +, 5–20% inhibition; +−, 30–50% inhibition; +++, 75–90% inhibition. A typical interaction assay resulted in 40 ± 8% of Mϕ ingesting platelets aged in the absence of plasma after a 10-min assay and 38 ± 6% after 40 min for platelets aged in the presence of plasma, in contrast to BOWES where the level of ingestion was 30 ± 12% and 20 ± 6%, respectively.

Platelets aged in plasma	Concentration	Mϕ (human)		BOWES (human)[a]		TEPM (mouse)[b]	HUVEC (human)[c]
		−	+	−	+		
EGTA	1 mM	−	ND[d]	−	ND	ND	ND
RGDS	1 mM	−	−	−	−	−	−
RGES	1 mM	−	−	−	−	−	−
Phospho-L-serine	1 mM	−	−	−	−	−	−
N-Acetyl glucosamine	20 mM	−	−	−	−	−	−
N-Acetyl galactosamine	20 mM	−	−	−	−	−	−
Poly-inositol	100 μg/ml	++	++	++	++	++	++
Poly-cytidine	100 μg/ml	−	−	−	−	−	−
Fucoidan	100 μg/ml	+++	+++	+++	+++	+++	+++
Dextran	100 μg/ml	−	ND	−	ND	ND	ND
mAb 2F8 (anti-scavenger receptor)	10 μg/ml	NA[e]	NA	NA	NA	++	NA
mAb G-1 (P-selectin blocking Ab)	10 μg/ml	+	ND	+	ND	ND	ND
mAb G-1 + 100 μg/ml poly-inositol	10 μg/ml	++	ND	++	ND	ND	ND
mAb G-1 + 1 mM EGTA	10 μg/ml	+	ND	+	ND	ND	ND
mAb CRC81 (anti-P-selectin)	10 μg/ml	−	ND	−	ND	ND	ND

[a] BOWES, Bowes melanoma cells.
[b] TEPM, thioglycollate-elicited peritoneal macrophages.
[c] HUVEC, human umbilical vein endothelial cells.
[d] ND, not done.
[e] NA, not applicable.

ability to aggregate in response to weak agonists such as ADP and failed to adhere and spread on collagen coated surfaces.

Given that serum is a rich source of survival factors for granulocytes, with which platelets have much in common, we reasoned that the physiological milieu of platelets, plasma, was likely to also contain factors capable of retarding their programmed death. We therefore sought evidence of accelerated programmed cell death when platelets were cultured in the

absence of plasma, finding that they not only exhibited an accelerated loss of function but also displayed many features in common with programmed death of granulocytes. Such changes included morphological evidence of cytoplasmic condensation and cell surface expression of the "eat me" signal phosphatidylserine (52) and granule components such as P-selectin. Importantly, however, there was strong evidence that such aged platelets retained plasma membrane integrity because there was no detectable release of the cytoplasmic marker enzyme lactate dehydrogenase, nor did the aged cells admit actin-binding phalloidin-FITC. Furthermore, aged platelets were recognized and ingested by all professional and semi-professional phagocytes tested by a mechanism in which phagocyte scavenger receptors predominated.

An important conclusion to be drawn from these studies is that constitutive death in plasma-deprived platelets represents a caspase-independent program for phagocyte clearance of effete cells. Not only did broad spectrum caspase inhibitors fail to affect all phenomena associated with platelet death, including recognition by phagocytes, but there was no evidence that caspase pseudo-substrates were cleaved. Given earlier studies in various models of apoptotic cell death, in which the display of cell surface "eat me" signals such as phosphatidylserine exposure appears to be mediated by caspases (8, 40, 42), we believe that demonstration of a complete caspase-independent program for cell death and clearance is an important addition to examples of caspase-independent cell death where recognition of such cells by phagocytes has not been previously studied (53–56).

With regard to platelet cell death, these studies also complement recent reports suggesting that ionomycin stimulation of platelets, a model of activation-induced cell death, can recapitulate many of the features found in apoptosis (19, 46). Although there is some evidence that caspases may participate in platelet activation (57), Wolf *et al.* (46) suggest that the effects of ionomycin on platelets are independent of caspases because calpains disable activation by partial cleavage of their prodomains. Similar mechanisms could be at play in constitutive platelet cell death given its independence from caspases despite the presence of cytochrome *c* in supernatants from cell lysates, which can activate caspases (46), and the remarkably similar evidence of partial caspase-3 cleavage on Western blot. This possibility is now under active investigation, especially as further studies are clearly required to characterize the caspase-independent mechanisms by which aged platelets express PS because this was not affected by the calpain inhibitors acetyl-leucyl-leucylnorleucinal and calpeptin.

It will also be important to define in more detail the molecular mechanisms by which phagocyte class A scavenger receptors mediate recognition and ingestion of aged platelets, as clearly indicated by the inhibitor studies presented in this report. Although the role of the scavenger receptor has already been implicated in the clearance of apoptotic thymocytes by mouse macrophages (58) the ligands displayed by dying cells, which lead to their recognition by scavenger receptors, are currently unknown. Furthermore, although PS is widely recognized as an "eat me" signal (52), it is not properly understood why various phagocyte types will apparently ignore PS in favor of other yet to be characterized signals for ingestion (59).

Because constitutive death in plasma-deprived platelets was caspase-independent and could not be assessed for typical nuclear changes because these cells have no nucleus, we feel it is not appropriate to label this form of cell death as "apoptosis." However, we believe that the data indicate that platelets can undergo a form of programmed cell death that can be regulated by exogenous influences, in particular plasma-derived survival factors. In keeping with this, we observed that the return of plasma-deprived platelets to plasma slowed the phenomena of cell death, most notably returning the rate of loss of aggregation and levels of PS exposure to that observed for platelets cultured in plasma. Ongoing work is directed at the biochemical characterization of the survival activity present in plasma because a range of candidate cytokine survival factors could not substitute for plasma. Nevertheless it should be emphasized that plasma deprivation appeared merely to accelerate a constitutive death program that was already active in platelets at 37 °C and evident after 18–24 h of culture in plasma. However, study of constitutive death in plasma-replete platelets will be difficult because our preliminary work demonstrated progressive loss of platelets from populations cultured for >24 h, presumably reflecting relatively rapid secondary necrosis of that proportion of cultured platelets undergoing programmed death each day.

In conclusion, we have provided *in vitro* evidence that human platelets can undergo a constitutive program of cell death that was caspase-independent and that resulted in the specific recognition of effete cells by phagocytes employing the scavenger receptor as a recognition mechanism. Although our findings have potentially important implications for understanding platelet kinetics and the related pathogenesis of thrombotic and bleeding disorders, no firm conclusions on *in vivo* relevance can be drawn from the current data. Nevertheless, these data raise the exciting prospect that platelet lifespan and clearance is amenable to exogenous regulation for therapeutic purposes.

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